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<th>Title</th>
<th>The Effect of Pain on Cognitive Function: Preclinical and Clinical Investigations</th>
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The Effect of Pain on Cognitive Function: Preclinical and Clinical Investigations

Orla Moriarty, B.Sc.
Pharmacology and Therapeutics, School of Medicine
NCBES Neuroscience Cluster and Centre for Pain Research
National University of Ireland, Galway

Supervisors
Dr. David P. Finn
Pharmacology and Therapeutics, School of Medicine
National University of Ireland, Galway

Dr. Brian E. McGuire
School of Psychology
National University of Ireland, Galway

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Abstract

Chronic pain is associated with high social and economic costs due to its disabling physical and psychological effects. Despite some evidence that chronic pain negatively affects cognitive function, little is known about the mechanisms by which pain and cognition interact and influence one another. Theories based on the existing literature suggest that pain may alter the accessibility of cognitive resources or may be associated with changes in neuroplasticity and neurochemistry, which may in turn impact on cognition. A role for age in the interaction between pain and cognition has also been suggested. The aims of the work presented in this thesis were to investigate the relationship between pain and cognitive function, and to elucidate potential mechanisms underlying this relationship, using a translational approach involving preclinical and clinical models.

The L5–L6 spinal nerve ligation (SNL) and complete Freund’s adjuvant models of chronic neuropathic and inflammatory pain, respectively, were employed in young and mid-aged rats and cognitive performance was assessed using behavioural tests of spatial learning, spatial and recognition memory and cognitive flexibility. A novel behavioural paradigm, which used air-puff to induce passive avoidance, was also developed and used to test aversive learning and memory in rat models of chronic pain. HPLC, immunohistochemistry and western immunoblotting were used to investigate the neural mechanisms underlying the relationship between chronic pain and cognitive function, specifically the role of the monoaminergic system and that of the synaptic protein synaptophysin as a potential indicator of synaptic plasticity. Cognitive function was also examined in a clinical sample of chronic neuropathic or radicular pain patients, in the domains of verbal memory, spatial memory, attention and executive function.

The studies provide some evidence for deficits in cognitive performance associated with the SNL model of neuropathic pain, which were dependent on age and on the cognitive task. Furthermore, the studies identify pain-related alterations in synaptophysin expression in regions such as the hippocampus and the prefrontal and amygdaloid cortices, which may contribute to the observed impairments in cognitive behaviour. Alterations in monoaminergic transmission were also identified in the SNL model in mid-aged rats. In the clinical study, chronic pain patients were found to perform poorly compared with controls on measures of intelligence and memory, and showed an altered pattern of responding in tests of attention. Significant interactions between these cognitive outcomes and age were also observed, highlighting the influence of age on the relationship between chronic pain and cognition.

In conclusion, these results expand on the previous literature suggesting that chronic pain is associated with impaired cognitive function, and suggest the involvement of neurochemical and neuroplastic mechanisms.
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"You are braver than you believe, stronger than you seem, and smarter than you think."
– from a story by A. A. Milne
Author’s Declaration

I hereby declare that the work presented in this thesis was carried out in accordance with the regulations of the National University of Ireland, Galway. The research is original and entirely my own work, except where explicitly noted in the text. The thesis or any part thereof has not been submitted to the National University of Ireland, Galway or to any other institution in connection with any other academic award. Any views expressed herein are those of the author.

Signed: ___________________________ Date: ______________
## Contents

Abstract i

Acknowledgements ii

Author’s Declaration v

Contents vii

List of Figures xv

List of Tables xxiii

List of Abbreviations xxv

List of Publications and Proceedings xxxi

1 General Introduction 1

1.1 Evidence for pain-related cognitive impairment 4

1.1.1 Clinical studies 4

1.1.1.1 Attention 5

1.1.1.2 Learning, memory and general cognition 16

1.1.1.3 Speed of information processing and psychomotor ability 17

1.1.1.4 Executive function 17

1.1.2 Preclinical studies 19

1.1.3 Limitations of the current literature 24

1.2 Potential mechanisms involved in pain-related cognitive impairment 28

1.2.1 Shared neuroanatomy in pain and cognition 29

1.2.2 Brain morphology and cellular alterations in pain and cognition 33

1.2.3 Synaptic plasticity in pain and cognitive impairment 36

1.2.4 Neurotransmitters and receptors in pain and cognition 39

1.2.4.1 Glutamate 39
## CONTENTS

1.2.4.2 GABA .................................................. 40  
1.2.4.3 Acetylcholine ................................. 41  
1.2.4.4 Endocannabinoids .......................... 41  
1.2.4.5 Aspartate ...................................... 42  
1.2.4.6 Opioids ........................................ 43  
1.2.4.7 Monoamines ................................. 44  
1.2.5 Glial cells and cytokines in pain and cognition .......... 45  
1.2.6 Enzymes in pain and cognition ..................... 47  
1.2.7 Neurotrophic factors in pain and cognition .......... 48  
1.3 The effects of analgesic treatments for chronic pain on cognitive function ........................................ 50  
1.4 The influence of ageing on pain and cognition ............. 54  
1.5 Overall objectives and experimental approach ................. 57  
1.5.1 Rationale for rodent pain models used .................. 58  
1.5.2 Rationale for rodent behavioural cognitive tests used ... 59  
1.5.3 Rationale for the investigation of synaptophysin and the monoaminergic system ................................ 59  
1.5.4 Rationale for investigation of the effects of amitriptyline . 60  
1.5.5 Rationale for selection of clinical participants and pain and cognitive measures ............................. 61  

2 Materials and Methods ............................. 63  
2.1 Materials ........................................ 63  
2.1.1 Animal husbandry materials ...................... 63  
2.1.2 Surgical procedure materials .................... 63  
2.1.3 *In vivo* testing equipment ...................... 64  
2.1.4 Drugs and drug administration .................... 65  
2.1.5 Transcardial perfusion ................................ 65  
2.1.6 Histology and immunohistochemistry ................ 66  
2.1.7 HPLC ........................................ 66  
2.1.8 Western blotting .................................. 67  
2.1.9 Clinical study materials .......................... 67  
2.1.10 Computer software ................................ 68  
2.2 Methods .......................................... 68  
2.2.1 Animal experimental subjects ..................... 68  
2.2.2 Animal models of chronic pain .................... 69  
2.2.2.1 Spinal nerve ligation surgery .................. 69
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.2.2</td>
<td>Complete Freund’s adjuvant injection</td>
<td>70</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Physiological and behavioural characteristics of chronic pain models</td>
<td>72</td>
</tr>
<tr>
<td>2.2.3.1</td>
<td>Measurement of hindpaw oedema</td>
<td>72</td>
</tr>
<tr>
<td>2.2.3.2</td>
<td>Behavioural measurement of sensory responses</td>
<td>72</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Behavioural measurements of cognitive function</td>
<td>79</td>
</tr>
<tr>
<td>2.2.4.1</td>
<td>Novel object recognition test</td>
<td>79</td>
</tr>
<tr>
<td>2.2.4.2</td>
<td>T-maze spontaneous alternation</td>
<td>82</td>
</tr>
<tr>
<td>2.2.4.3</td>
<td>Morris water maze</td>
<td>84</td>
</tr>
<tr>
<td>2.2.4.4</td>
<td>Passive avoidance</td>
<td>88</td>
</tr>
<tr>
<td>2.2.5</td>
<td>Drugs</td>
<td>91</td>
</tr>
<tr>
<td>2.2.6</td>
<td>Animal sacrifice and tissue collection</td>
<td>92</td>
</tr>
<tr>
<td>2.2.6.1</td>
<td>Transcardial perfusion</td>
<td>92</td>
</tr>
<tr>
<td>2.2.6.2</td>
<td>Tissue collection</td>
<td>93</td>
</tr>
<tr>
<td>2.2.7</td>
<td>Histology and immunohistochemistry</td>
<td>94</td>
</tr>
<tr>
<td>2.2.7.1</td>
<td>Isolectin B4 (IB4) staining</td>
<td>94</td>
</tr>
<tr>
<td>2.2.7.2</td>
<td>Synaptophysin immunohistochemistry</td>
<td>94</td>
</tr>
<tr>
<td>2.2.8</td>
<td>Western immunoblotting</td>
<td>96</td>
</tr>
<tr>
<td>2.2.9</td>
<td>High-performance liquid chromatography with electrochemical detection for measurement of brain monoamines</td>
<td>97</td>
</tr>
<tr>
<td>2.2.10</td>
<td>Human participants</td>
<td>99</td>
</tr>
<tr>
<td>2.2.11</td>
<td>Participant demographics</td>
<td>101</td>
</tr>
<tr>
<td>2.2.12</td>
<td>Pain measures</td>
<td>101</td>
</tr>
<tr>
<td>2.2.13</td>
<td>Depression and anxiety measures</td>
<td>101</td>
</tr>
<tr>
<td>2.2.13.1</td>
<td>Patient health questionnaire</td>
<td>101</td>
</tr>
<tr>
<td>2.2.13.2</td>
<td>Spielberger State Anxiety Inventory</td>
<td>102</td>
</tr>
<tr>
<td>2.2.14</td>
<td>General intellect</td>
<td>102</td>
</tr>
<tr>
<td>2.2.15</td>
<td>Cognitive measures</td>
<td>103</td>
</tr>
<tr>
<td>2.2.15.1</td>
<td>Verbal memory</td>
<td>103</td>
</tr>
<tr>
<td>2.2.15.2</td>
<td>Spatial memory</td>
<td>104</td>
</tr>
<tr>
<td>2.2.15.3</td>
<td>Attention/vigilance</td>
<td>104</td>
</tr>
<tr>
<td>2.2.15.4</td>
<td>Executive function</td>
<td>105</td>
</tr>
<tr>
<td>2.2.15.5</td>
<td>Perceived impact of pain</td>
<td>106</td>
</tr>
<tr>
<td>2.2.16</td>
<td>Statistical analyses</td>
<td>106</td>
</tr>
</tbody>
</table>
### 3 Characterisation of Cognitive Behaviour in the SNL Model of Neuropathic Pain in Young Rats

**3.1 Introduction** ................................................. 107

**3.2 Methods** .................................................. 110

- **3.2.1 Animals** .............................................. 110
- **3.2.2 Experimental protocol** ................................. 110
- **3.2.3 Statistical analysis** ................................. 111

**3.3 Results** .................................................. 113

- **3.3.1 Sensory testing** ....................................... 113
  - **3.3.1.1 Mechanical allodynia: von Frey testing** ........ 113
  - **3.3.1.2 Thermal hypersensitivity: Hargreaves testing** 118
- **3.3.2 Cognitive testing** ................................... 120
  - **3.3.2.1 Novel object recognition** ......................... 120
  - **3.3.2.2 T-maze** ........................................... 124
  - **3.3.2.3 Morris water maze** ............................... 126
- **3.3.3 IB4 staining** .......................................... 133

**3.4 Discussion** .................................................. 136

### 4 Assessment of Aversive Learning and Memory in Models of Chronic Pain: Development of a Novel Passive Avoidance Paradigm

**4.1 Introduction** ................................................. 143

**4.2 Methods** .................................................. 146

- **4.2.1 Animals** .............................................. 146
- **4.2.2 Experimental protocols** ................................. 147
- **4.2.3 Statistical analysis** .................................. 150

**4.3 Results** .................................................. 150

- **4.3.1 Experiment 1: Use of ultrasound in the passive-avoidance paradigm** ................................. 150
- **4.3.2 Experiment 2: Use of air-puff in the passive-avoidance paradigm and pharmacological validation of the response using scopolamine** ................................. 150
- **4.3.3 Experiment 3: Assessment of aversive learning in rat models of chronic neuropathic and inflammatory pain** ................................. 153
  - **4.3.3.1 Sensory testing** .................................. 153
  - **4.3.3.2 IB4 staining** ...................................... 160
  - **4.3.3.3 Passive-avoidance testing** ....................... 161

**4.4 Discussion** .................................................. 163
5 Characterisation of Cognitive Behaviour in the SNL Model in Mid-Aged Rats, and Synaptophysin Expression in the Hippocampus and PFC 171
5.1 Introduction ................................................. 171
5.2 Methods ..................................................... 174
  5.2.1 Animals .................................................. 174
  5.2.2 Experimental protocol .............................. 174
  5.2.3 Statistical analysis .................................... 176
5.3 Results ....................................................... 176
  5.3.1 Sensory testing ......................................... 176
    5.3.1.1 von Frey test ..................................... 176
    5.3.1.2 Hargreaves test .................................. 178
    5.3.1.3 Acetone-drop test ................................. 179
  5.3.2 Cognitive testing ....................................... 181
    5.3.2.1 Novel object recognition ....................... 181
    5.3.2.2 Air-puff passive avoidance ..................... 185
    5.3.2.3 Morris water maze .............................. 186
  5.3.3 Synaptophysin immunohistochemistry .............. 196
5.4 Discussion .................................................. 200

6 Investigation of the Effects of Amitriptyline in the SNL Model in Mid-Aged Rats on Behaviour, Synaptophysin Expression and Monoamine Levels 207
6.1 Introduction ............................................... 207
6.2 Methods .................................................... 209
  6.2.1 Animals ............................................... 209
  6.2.2 Experimental protocol ............................ 210
  6.2.3 Statistical analysis ................................ 211
6.3 Results ..................................................... 213
  6.3.1 Amitriptyline dosing ................................ 213
  6.3.2 Sensory testing ....................................... 217
    6.3.2.1 von Frey test .................................. 217
    6.3.2.2 Hargreaves test ................................ 217
    6.3.2.3 Acetone-drop test ............................. 219
  6.3.3 Cognitive testing ..................................... 222
    6.3.3.1 Novel object recognition .................... 222
    6.3.3.2 Morris water maze ............................. 225
6.3.4 Post-mortem analysis .............................................. 231
  6.3.4.1 Synaptophysin western immunoblotting .......... 232
  6.3.4.2 Assessment of brain and spinal cord monoamine
          levels using HPLC ........................................... 235
6.4 Discussion .............................................................. 243

7 Investigation of Cognitive Performance in a Clinical Sample of
Chronic Pain Patients ................................................. 255
7.1 Introduction .............................................................. 255
7.2 Materials and methods ................................................ 259
  7.2.1 Experimental design .............................................. 259
  7.2.2 Participants ...................................................... 259
  7.2.3 Materials .......................................................... 260
    7.2.3.1 Demographic questionnaire outcome measures .. 260
    7.2.3.2 Pain questionnaire outcome measures .......... 261
    7.2.3.3 Psychological questionnaire outcome measures .. 262
    7.2.3.4 Cognitive function outcome measures .......... 263
  7.2.4 Procedure ........................................................ 265
  7.2.5 Statistical design and analysis ............................... 266
7.3 Results .................................................................. 268
  7.3.1 Phase I: Analysis by group ....................................... 268
    7.3.1.1 Descriptive and inferential statistics .......... 268
    7.3.1.2 Correlations ................................................. 272
    7.3.1.3 Hypothesis testing .......................................... 272
    7.3.1.4 Intelligence .................................................. 273
    7.3.1.5 Immediate verbal memory ......................... 277
    7.3.1.6 Delayed verbal memory ................................. 277
    7.3.1.7 Spatial memory ............................................. 281
    7.3.1.8 Attention .................................................... 281
    7.3.1.9 Psychomotor speed ....................................... 285
    7.3.1.10 Executive function ...................................... 285
  7.3.2 Phase II: Analysis by pain variable ......................... 286
    7.3.2.1 Descriptive statistics .................................... 286
    7.3.2.2 Correlations ............................................... 287
    7.3.2.3 Hypothesis testing ....................................... 293
7.4 Discussion ................................................................ 300
  7.4.1 Participant characteristics ..................................... 300
CONTENTS

7.4.2 Cognitive measures ........................................ 303
  7.4.2.1 Phase I ........................................... 303
  7.4.2.2 Phase II ........................................... 307
7.4.3 Limitations .................................................. 308
7.4.4 Conclusions .................................................. 309

8 General Discussion ................................................ 311

Bibliography ......................................................... 325

Appendix A Buffers and Solutions .................................. 367
  A.1 Transcardial perfusion ....................................... 367
  A.2 Histology and immunohistochemistry ......................... 369
  A.3 Western immunoblotting ..................................... 370
  A.4 High-performance liquid chromatography (HPLC) .......... 373

Appendix B Supplementary Data .................................... 375
  B.1 Supplementary results for Chapter 4 ....................... 376
  B.2 Supplementary results for Chapter 5 ....................... 382
  B.3 Supplementary results for Chapter 6 ....................... 387

Appendix C Ethovision® System .................................... 389
  C.1 Ethovision® 3.1 Quick Start Guide ......................... 391
  C.2 Ethovision® XT Novel Object Test ......................... 426
  C.3 Ethovision® XT Morris Water Maze ......................... 437

Appendix D ImageJ System .......................................... 449
  D.1 Using ImageJ for western immunoblotting .................. 449
  D.2 Using ImageJ for immunohistochemistry .................... 451

Appendix E Clinical Study Materials ............................... 455
  E.1 Patient questionnaire ....................................... 456
  E.2 Test forms ............................................... 466
  E.3 Invitation letter for patient volunteers .................... 475
  E.4 Advertisement for healthy volunteers ....................... 477
  E.5 Information sheet for patient volunteers ................... 478
  E.6 Information sheet for healthy volunteers ................... 481

xiii
List of Figures

1.1 The most common causes of pain ........................................ 2
1.2 Brain regions involved in pain perception .......................... 30
1.3 Transmembrane behaviour of synaptophysin ..................... 39
1.4 Pain-related cognitive impairment model ......................... 49
1.5 Effect of spared-nerve injury (SNI) on spatial reversal in the Morris water maze .................................................... 56
2.1 SNL surgery .................................................................. 71
2.2 Plethysmometer set-up .................................................. 73
2.3 Lateral malleolus of the rat hindpaw ................................. 73
2.4 von Frey mechanical allodynia testing .............................. 76
2.5 Hargreaves test ............................................................. 78
2.6 Novel object recognition testing ...................................... 81
2.7 T-maze apparatus .......................................................... 83
2.8 Morris water maze .......................................................... 85
2.9 Representative chromatogram .......................................... 98
2.10 Manikin diagram ........................................................... 102
2.11 Spatial span board .......................................................... 104
2.12 Wisconsin card-sorting test ............................................. 105
3.1 Experimental timeline .................................................... 112
3.2 Effect of SNL surgery on mechanical response threshold in ipsilateral paws .................................................. 114
3.3 Effect of SNL surgery on the % response to mechanical stimulation of the ipsilateral hindpaw ........................................... 115
3.4 Mechanical response threshold in the ipsilateral and contralateral paws following SNL surgery ............................................. 115
3.5 Effect of SNL surgery on mechanical response threshold in the contralateral paws .......................................................... 116
3.6 Mechanical response threshold in ipsilateral and contralateral paws following sham surgery ............................................. 116

xv
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7</td>
<td>Effect of SNL surgery on ipsilateral hindpaw withdrawal latency in the Hargreaves test</td>
<td>118</td>
</tr>
<tr>
<td>3.8</td>
<td>Effect of SNL surgery on paw withdrawal latency in the Hargreaves test in SNL rats in ipsilateral and contralateral hindpaws</td>
<td>119</td>
</tr>
<tr>
<td>3.9</td>
<td>Paw withdrawal latency in the Hargreaves test in contralateral paws of sham and SNL rats</td>
<td>119</td>
</tr>
<tr>
<td>3.10</td>
<td>Paw withdrawal latency in the Hargreaves test in ipsilateral and contralateral paws of sham control rats</td>
<td>119</td>
</tr>
<tr>
<td>3.11</td>
<td>Novel object recognition in sham and SNL rats</td>
<td>121</td>
</tr>
<tr>
<td>3.12</td>
<td>Total duration of object exploration in novel object recognition</td>
<td>122</td>
</tr>
<tr>
<td>3.13</td>
<td>Duration of general and exploratory behaviours in SNL and sham rats during novel object habituation</td>
<td>122</td>
</tr>
<tr>
<td>3.14</td>
<td>Frequency of general and exploratory behaviours in SNL and sham rats during novel object habituation</td>
<td>123</td>
</tr>
<tr>
<td>3.15</td>
<td>Locomotor activity of SNL and sham rats during novel object habituation</td>
<td>123</td>
</tr>
<tr>
<td>3.16</td>
<td>% spontaneous alternation in the T-maze in SNL and sham rats</td>
<td>124</td>
</tr>
<tr>
<td>3.17</td>
<td>% errors in the T-maze alternation in SNL and sham rats</td>
<td>124</td>
</tr>
<tr>
<td>3.18</td>
<td>% trials omitted in the T-maze in SNL and sham rats</td>
<td>125</td>
</tr>
<tr>
<td>3.19</td>
<td>Average latency to arm choice in the T-maze alternation task in SNL and sham rats</td>
<td>125</td>
</tr>
<tr>
<td>3.20</td>
<td>Total number of arm entries in the T-maze alternation task in SNL and sham rats</td>
<td>125</td>
</tr>
<tr>
<td>3.21</td>
<td>Locomotor activity of SNL and sham rats in the T-maze alternation task</td>
<td>126</td>
</tr>
<tr>
<td>3.22</td>
<td>Latency to get onto the platform in sham and SNL rats in the cued trial of the Morris water maze</td>
<td>127</td>
</tr>
<tr>
<td>3.23</td>
<td>Path length to locate the platform in sham and SNL rats in the cued trial of the Morris water maze</td>
<td>128</td>
</tr>
<tr>
<td>3.24</td>
<td>Swim speed of sham and SNL rats in the cued trial of the Morris water maze</td>
<td>128</td>
</tr>
<tr>
<td>3.25</td>
<td>Latency to get onto the platform in sham and SNL rats in the acquisition phase of the Morris water maze</td>
<td>129</td>
</tr>
<tr>
<td>3.26</td>
<td>Path length to locate the platform in sham and SNL rats in the acquisition phase of the Morris water maze</td>
<td>130</td>
</tr>
<tr>
<td>3.27</td>
<td>Swim speed of sham and SNL rats in the acquisition phase of the Morris water maze</td>
<td>130</td>
</tr>
</tbody>
</table>
3.28 % time spent in arena zones during the probe trial in sham and SNL groups .......................................................... 131
3.29 % distance moved in arena zones during the probe trial in sham and SNL groups .......................................................... 132
3.30 Total distance moved by sham and SNL rats during the probe trial .......................................................... 132
3.31 Swim speed of sham and SNL rats during the probe trial .......................................................... 133
3.32 Representative images of IB4-stained sections from regions L4–L6 of the spinal cord of control and SNL rats .......................................................... 134
3.33 Quantification of IB4 staining intensity .......................................................... 135

4.1 Chemical structure of scopolamine .......................................................... 146
4.2 Timelines for the three experiments described in Chapter 4 .......................................................... 149
4.3 Effect of ultrasound exposure on behaviour in passive-avoidance paradigm .......................................................... 151
4.4 Passive-avoidance response induced by air-puff, and effect of scopolamine .......................................................... 152
4.5 Extinction trials for air-puff-induced passive avoidance response .......................................................... 153
4.6 Effect of SNL surgery on the mechanical response threshold of the ipsilateral hindpaw .......................................................... 154
4.7 Effect of CFA injection on the mechanical response threshold of the ipsilateral hindpaw .......................................................... 155
4.8 Effect of SNL surgery on ipsilateral hindpaw withdrawal latency in the Hargreaves test .......................................................... 156
4.9 Effect of CFA injection on withdrawal latency of the ipsilateral hindpaw in the Hargreaves test .......................................................... 156
4.10 Effect of SNL surgery on the total number of hindpaw withdrawal responses following acetone application .......................................................... 157
4.11 Effect of CFA injection on the total number of hindpaw withdrawal responses following acetone application .......................................................... 158
4.12 Effect of intraplantar CFA injection on ipsilateral paw volume .......................................................... 159
4.13 Effect of intraplantar CFA injection on ipsilateral paw circumference .......................................................... 159
4.14 Representative images of IB4-stained sections from regions L4–L6 of the spinal cord of control and SNL rats .......................................................... 160
4.15 Passive-avoidance response following air-puff in SNL and sham rats .......................................................... 161
4.16 Passive-avoidance response following air-puff in CFA-injected and control rats .......................................................... 162
5.1 Experimental timeline ............................................. 177
5.2 Effect of SNL surgery on the mechanical response threshold of the ipsilateral hindpaw ............................................. 178
5.3 Effect of SNL surgery on ipsilateral hindpaw withdrawal latency in the Hargreaves test ............................................. 179
5.4 Effect of SNL surgery on the total number of hindpaw withdrawal responses following acetone application to the ipsilateral paw ... 180
5.5 Novel object recognition in sham and SNL rats ................... 182
5.6 Total duration of object exploration in novel object recognition .. 183
5.7 Locomotor activity of SNL and sham rats during the novel object habituation trial ....................................................... 183
5.8 Duration of general and exploratory behaviours in SNL and sham rats during novel object habituation trial ......................... 184
5.9 Frequency of general and exploratory behaviours in SNL and sham rats during novel object habituation phase ......................... 184
5.10 Apparent orientation bias in the novel object recognition test in SNL rats ................................................................. 185
5.11 Passive-avoidance response following air-puff in SNL and sham control rats .............................................................. 186
5.12 Latency to get onto the platform in mid-aged sham and SNL rats in the cued trial of the Morris water maze ......................... 187
5.13 Path length to locate the platform in sham and SNL rats in the acquisition phase of the Morris water maze ......................... 188
5.14 % distance moved in arena zones during the probe trial in sham and SNL groups ........................................................... 189
5.15 % distance moved in the SW quadrant (old platform location) by sham and SNL rats (data averaged over 4 trials per day) ......... 191
5.16 % time spent in the SW quadrant (old platform location) by sham and SNL rats (data averaged over 4 trials per day) ............. 192
5.17 % distance moved in the SW quadrant (old platform location) by sham and SNL rats (data for the first trial on each day) ........... 192
5.18 % distance moved in the NE quadrant (new platform location) by sham and SNL rats (data averaged over 4 trials per day) ......... 193
5.19 % time spent in the NE quadrant (new platform location) by sham and SNL rats (data averaged over 4 trials per day) ............. 194
5.20 % distance moved in the NE quadrant (new platform location) by sham and SNL rats (data for the first trial on each day) ......... 194
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.21</td>
<td>% distance moved in arena zones during the reversal probe trial in</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>sham and SNL groups</td>
<td></td>
</tr>
<tr>
<td>5.22</td>
<td>Representative images of reversal tracks for sham and SNL rats</td>
<td>196</td>
</tr>
<tr>
<td>5.23</td>
<td>Density of synaptophysin staining in mPFC of sham and SNL rats</td>
<td>197</td>
</tr>
<tr>
<td>5.24</td>
<td>Density of synaptophysin staining in the CA1 region of the hippocampus of</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>sham and SNL rats</td>
<td></td>
</tr>
<tr>
<td>5.25</td>
<td>Representative images of synaptophysin immunostained sections of</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>the mPFC</td>
<td></td>
</tr>
<tr>
<td>5.26</td>
<td>Representative images of synaptophysin immunostained sections of</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>the CA1 region of the hippocampus</td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>Chemical structure of amitriptyline</td>
<td>207</td>
</tr>
<tr>
<td>6.2</td>
<td>Experimental timeline</td>
<td>212</td>
</tr>
<tr>
<td>6.3</td>
<td>Amitriptyline dosing in sham and SNL rats vs. time post surgery</td>
<td>215</td>
</tr>
<tr>
<td>6.4</td>
<td>Body weight of rats expressed per week post surgery</td>
<td>216</td>
</tr>
<tr>
<td>6.5</td>
<td>Effect of treatment on the mechanical response threshold of the</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>ipsilateral hindpaw</td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>Effect of treatment on ipsilateral hindpaw withdrawal latency in</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>the Hargreaves test</td>
<td></td>
</tr>
<tr>
<td>6.7</td>
<td>Effect of treatment on the total number of hindpaw withdrawal responses</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td>following acetone application to the ipsilateral paw</td>
<td></td>
</tr>
<tr>
<td>6.8</td>
<td>Effect of treatment on novel object recognition</td>
<td>223</td>
</tr>
<tr>
<td>6.9</td>
<td>Total duration of object exploration in novel object recognition</td>
<td>223</td>
</tr>
<tr>
<td>6.10</td>
<td>Locomotor activity during the novel object habituation phase</td>
<td>224</td>
</tr>
<tr>
<td>6.11</td>
<td>Duration of general and exploratory behaviours during novel object</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>habituation phase</td>
<td></td>
</tr>
<tr>
<td>6.12</td>
<td>Apparent orientation bias in the novel object recognition test</td>
<td>225</td>
</tr>
<tr>
<td>6.13</td>
<td>Latency to get onto the platform in the cued trial of the Morris water maze</td>
<td>226</td>
</tr>
<tr>
<td>6.14</td>
<td>Path length to locate the platform in the acquisition phase of the</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>Morris water maze</td>
<td></td>
</tr>
<tr>
<td>6.15</td>
<td>% distance moved in arena zones during the probe trial</td>
<td>228</td>
</tr>
<tr>
<td>6.16</td>
<td>% distance moved in the SW quadrant (old platform location)</td>
<td>229</td>
</tr>
<tr>
<td>6.17</td>
<td>% distance moved in the NE quadrant (new platform location)</td>
<td>230</td>
</tr>
<tr>
<td>6.18</td>
<td>% distance moved in arena zones during the reversal probe trial in</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>sham and SNL groups</td>
<td></td>
</tr>
<tr>
<td>6.19</td>
<td>Representative western immunoblot image</td>
<td>232</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

6.20 Relative expression of synaptophysin in discrete brain regions . . 233
6.21 Lateralised relative expression of synaptophysin in the amygdaloid
cortex ......................................................... 234
6.22 Levels of monoamine transmitters, metabolites and turnovers in
the cerebral cortex ............................................. 236
6.23 Levels of monoamine transmitters and metabolites in the thalamus 239
6.24 Levels of monoamine transmitters, metabolites and turnovers in
the dorsal spinal cord ........................................... 241
6.25 Levels of serotonin in the hippocampus .............................. 242
6.26 Lateralised levels of HVA in the amygdaloid cortex ............... 242

7.1 FSIQ for old and young patients and controls ...................... 276
7.2 Verbal learning slope for old and young patients and controls . . . 278
7.3 Verbal recognition memory for old and young patients and controls 279
7.4 Spatial span reverse scores for old and young patients and controls 280
7.5 CPT hit responses for old and young patients and controls ........ 282
7.6 CPT false alarm responses for old and young patients and controls . 283
7.7 CPT random responses for old and young patients and controls . 284
7.8 CPT hit reaction times for old and young patients and controls . 286

B.1 Effect of SNL surgery on % withdrawal response to mechanical
stimulation with 4 g von Frey filament .................................. 376
B.2 Effect of SNL surgery on mechanical response threshold in ipsilat-
eral vs. contralateral paws ........................................... 376
B.3 Effect of SNL surgery on mechanical response threshold in con-
tralateral paws ......................................................... 376
B.4 Effect of sham surgery on mechanical response threshold in ipsilat-
eral and contralateral paws .......................................... 376
B.5 Effect of SNL surgery on withdrawal latency in ipsilateral vs. con-
tralateral paws ......................................................... 377
B.6 Effect of SNL surgery on withdrawal latency in contralateral paws 377
B.7 Effect of sham surgery on withdrawal latency in ipsilateral and
contralateral paws ....................................................... 377
B.8 Effect of SNL surgery on total number of responses to acetone in
ipsilateral vs. contralateral paws ...................................... 377
B.9 Effect of SNL surgery on total number of responses to acetone in
contralateral paws ....................................................... 378
B.10 Effect of sham surgery on total number of responses to acetone in
ipsilateral and contralateral paws ........................................ 378
B.11 Effect of CFA injection on % withdrawal response to mechanical
stimulation with 4 g von Frey filament ..................................... 379
B.12 Effect of CFA injection on mechanical response threshold in ipsi-
lateral and contralateral paws ............................................. 379
B.13 Effect of CFA injection on mechanical response threshold in con-
tralateral paws ........................................................................ 379
B.14 Effect of control needle insertion on mechanical response threshold
in ipsilateral and contralateral paws .......................................... 379
B.15 Effect of CFA injection on withdrawal latency in ipsilateral vs. con-
tralateral paws ....................................................................... 380
B.16 Effect of CFA injection on withdrawal latency in contralateral paws 380
B.17 Effect of control needle insertion on withdrawal latency in ipsilat-
eral vs. contralateral paws ..................................................... 380
B.18 Effect of CFA injection on number of responses in ipsilateral vs.
contralateral paws .................................................................. 380
B.19 Effect of CFA injection on withdrawal latency in contra-
lateral paws ........................................................................ 381
B.20 Effect of control needle insertion on number of responses in ipsilat-
eral vs. contralateral paws ..................................................... 381
B.21 Effect of SNL surgery on % withdrawal response to mechanical
stimulation with 4 g von Frey filament ...................................... 382
B.22 Effect of SNL surgery on mechanical response threshold in ipsilat-
eral vs. contralateral paws ..................................................... 382
B.23 Effect of SNL surgery on mechanical response threshold in con-
tralateral paws ....................................................................... 382
B.24 Effect of sham surgery on mechanical response threshold in ipsilat-
eral and contralateral paws .................................................... 382
B.25 Effect of SNL surgery on withdrawal latency in ipsilateral vs. con-
tralateral paws ....................................................................... 382
B.26 Effect of SNL surgery on withdrawal latency in contralateral paws 383
B.27 Effect of sham surgery on withdrawal latency in ipsilateral and
contralateral paws .................................................................. 383
B.28 Effect of SNL surgery on total number of responses to acetone in
ipsilateral vs. contralateral paws .............................................. 383
B.29 Effect of SNL surgery on total number of responses to acetone in contralateral paws ......................................................... 384
B.30 Effect of sham surgery on total number of responses to acetone in ipsilateral and contralateral paws ................................. 384
B.31 Latency to get onto platform in acquisition phase of Morris water maze ................................................................. 384
B.32 Average swim speed in acquisition phase of Morris water maze ................................................................. 384
B.33 % time in quadrants and platform zones in forward probe trial of Morris water maze .................................................. 385
B.34 % time spent in SW quadrant in trial 1 of reversal training in Morris water maze ...................................................... 385
B.35 % time spent in NE quadrant in trial 1 of reversal training ........ 385
B.36 Average swim speed in reversal training in Morris water maze ................................................................. 385
B.37 % time spent in quadrants and platform zones in reversal probe trial of Morris water maze ......................................... 386
B.38 Effect of treatment on % withdrawal response to 4 g von Frey filament in ipsilateral paws ..................................................... 387
B.39 Effect of treatment on mechanical response threshold in contralateral paws ............................................................ 387
B.40 Effect of treatment on paw withdrawal latency in contralateral paws ............................................................... 387
B.41 Effect of treatment on total number of contralateral paw withdrawals following acetone application .......................... 387
B.42 Latency to get onto platform in acquisition phase of Morris water maze ................................................................. 388
B.43 Average swim speed in acquisition phase of Morris water maze ................................................................. 388
B.44 % time spent in SW quadrant in reversal training in Morris water maze ............................................................... 388
B.45 % time spent in each quadrant during reversal probe trial in Morris water maze ............................................................... 388

E.1 Advertisement inviting members of the public to participate as controls in the study ................................................................. 477
List of Tables

1.1 Summary of clinical studies of chronic pain and cognition . . . . 15
1.2 Summary of commonly used animal models of chronic pain . . . 20
1.3 Preclinical animal studies . . . . . . . . . . . . . . . . . . . . . 23
1.4 Cognitive effects of analgesics . . . . . . . . . . . . . . . . . . . 53
2.1 Cued-learning trial patterns . . . . . . . . . . . . . . . . . . . . . 86
2.2 Release positions for Morris water maze acquisition-training trials 87
2.3 Participant selection criteria . . . . . . . . . . . . . . . . . . . . . 100
6.1 Fluid consumption, body weight and amitriptyline dose information 215
6.2 HPLC results for monoamine levels in discrete brain regions of 238
  sham/SNL rats treated with amitriptyline/water . . . . . . . . . . 238
6.3 Comparison of experimental parameters between Chapters 5 and 6 250
7.1 Demographic information . . . . . . . . . . . . . . . . . . . . . . 269
7.2 Psychological variables . . . . . . . . . . . . . . . . . . . . . . . . 270
7.3 Group comparisons of cognitive outcomes . . . . . . . . . . . . . 271
7.4 Bivariate correlation matrix of participant characteristics and cog- 275
  nitive outcomes . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 275
7.5 Hierarchical multiple regression predicting full-scale IQ . . . . . 276
7.6 Hierarchical multiple regression predicting verbal learning slope . 278
7.7 Hierarchical multiple regression predicting verbal recognition memory279
7.8 Hierarchical multiple regression predicting spatial span reversal . . 280
7.9 Hierarchical multiple regression predicting the number of hit re- 282
  sponses in the CPT . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 282
7.10 Hierarchical multiple regression predicting the number of false alarm 283
    responses in the CPT . . . . . . . . . . . . . . . . . . . . . . . . . . . 283
7.11 Hierarchical multiple regression predicting the number of random 284
    responses in the CPT . . . . . . . . . . . . . . . . . . . . . . . . . . . 284
7.12 Hierarchical multiple regression predicting hit reaction time in the 285
    CPT . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 285
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.13</td>
<td>Pain variables descriptives</td>
<td>287</td>
</tr>
<tr>
<td>7.14</td>
<td>Breakdown of patient medications</td>
<td>287</td>
</tr>
<tr>
<td>7.15</td>
<td>Correlation matrix of general patient characteristics and cognitive outcomes</td>
<td>290</td>
</tr>
<tr>
<td>7.16</td>
<td>Correlation matrix of patient-specific characteristics and cognitive outcomes</td>
<td>292</td>
</tr>
<tr>
<td>7.17</td>
<td>Hierarchical multiple regression predicting full-scale IQ with pain chronicity as an independent variable</td>
<td>293</td>
</tr>
<tr>
<td>7.18</td>
<td>Hierarchical multiple regression predicting Logical Memory I story unit recall with pain chronicity as an independent variable</td>
<td>294</td>
</tr>
<tr>
<td>7.19</td>
<td>Hierarchical multiple regression predicting Logical Memory II story unit recall with pain chronicity as an independent variable</td>
<td>295</td>
</tr>
<tr>
<td>7.20</td>
<td>Hierarchical multiple regression predicting hit response reaction time with pain chronicity as an independent variable</td>
<td>295</td>
</tr>
<tr>
<td>7.21</td>
<td>Hierarchical multiple regression predicting Logical Memory I story unit recall with number of painful areas as an independent variable</td>
<td>296</td>
</tr>
<tr>
<td>7.22</td>
<td>Hierarchical multiple regression predicting Logical Memory II story unit recall with number of painful areas as an independent variable</td>
<td>296</td>
</tr>
<tr>
<td>7.23</td>
<td>Hierarchical multiple regression predicting spatial span reversal with number of painful areas as an independent variable</td>
<td>297</td>
</tr>
<tr>
<td>7.24</td>
<td>Hierarchical multiple regression predicting the number of random responses on the CPT with number of painful areas as an independent variable</td>
<td>297</td>
</tr>
<tr>
<td>7.25</td>
<td>Hierarchical multiple regression predicting CPT D-prime score with Chronic Pain Grade as an independent variable</td>
<td>298</td>
</tr>
<tr>
<td>7.26</td>
<td>Hierarchical multiple regression predicting CPT D-prime score with pain-related disability as an independent variable</td>
<td>298</td>
</tr>
<tr>
<td>7.27</td>
<td>Hierarchical multiple regression predicting verbal memory retention with pain-related disability as an independent variable</td>
<td>299</td>
</tr>
<tr>
<td>7.28</td>
<td>Hierarchical multiple regression predicting the number of hit responses on the CPT with present pain intensity as an independent variable</td>
<td>299</td>
</tr>
</tbody>
</table>
List of Abbreviations

4CRT: four-choice reaction time
5CSRTT: five-choice serial reaction-time test
5-HIAA: 5-hydroxyindole-3-acetic acid
5-HT: 5-hydroxytryptamine
ACC: anterior cingulate cortex
ACR: American College of Rheumatology
AF488: Alexa Fluor 488
AIMS: Arthritis Impact Measurement Scales
ami: amitriptyline
AMPA: 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid
Amy/AMYG: amygdala
ANCOVA: analysis of covariance
ANOVA: analysis of variance
APS: ammonium persulphate
BB/Wor: biobreeding/Worcester
BDNF: brain-derived neurotrophic factor
$\beta$: standardised regression coefficient
$c_{\text{IS}}$: concentration of internal standard
$c_M$: concentration of monoamine or monoamine metabolite
CA1: cornu Ammonis 1
CALT: conditioned associative learning task
CAMKII: calmodulin-dependent kinase II
CCI: chronic constriction injury
CCSE: Cognitive Capacity Screening Exam
CFA: complete Freund’s adjuvant
$\chi^2_{(df)}$: chi-squared value with $df$ degrees of freedom
LIST OF ABBREVIATIONS

CNS: central nervous system
COMT: catechol-O-methyltransferase
CPG: Chronic Pain Grade
CPT-IP: Continuous Performance Test – Identical Pairs
CPT: Continuous Performance Test
CRPS: complex regional pain syndrome
CSF: cerebrospinal fluid
CVLT: California Verbal Learning Test
D: diameter
DA: dopamine
\( \Delta R^2 \): change in \( R^2 \)
D-KEFS: Delis-Kaplan Executive Function System
DOPAC: 3,4-dihydroxyphenylacetic acid
DRG: dorsal root ganglion
DVD: digital versatile disc
EC: endocannabinoid
EDTA: ethylenediaminetetraacetic acid
EEG: electroencephalography
EPSP: excitatory post-synaptic potential
ERK: extracellular-signal-regulated kinase
\( F_{(k-1,n-k)} \): F-test statistic with \( k \) groups and overall sample size \( n \)
fEPSP: field excitatory post-synaptic potential
FIQ: fibromyalgia impact questionnaire
FITC: fluorescein isothiocyanate
FM: fibromyalgia
fMRI: functional magnetic resonance imaging
\( f_R \): relative retention factor
FSIQ: full-scale intelligence quotient
GABA: gamma-aminobutyric acid
GTP\( \gamma \)S: guanosine 5\'{\textprime}-O-[gamma-thio]triphosphate
\( h \): height
$h_{IS}$: peak height for internal standard

$h_{M}$: peak height for monoamine or monoamine metabolite

Hipp: hippocampus

HPLC: high-performance liquid chromatography

HT: hypothalamus

HVA: homovanillic acid

IASP: International Association for the Study of Pain

IB4: isolectin B4

IC: insular cortex

IgG: immunoglobulin G

IGT: Iowa Gambling Task

IHS: International Headache Society

IL-1: interleukin 1

IL-1$\beta$: interleukin 1$\beta$

IL-6: interleukin 6

i.p.: intraperitoneal

IQ: intelligence quotient

$l$: length

L-DOPA: L-3,4-dihydroxyphenylalanine

LMA: locomotor activity

LSD: least significant difference

LTD: long-term depression

LTP: long-term potentiation

MATRICS: Measurement and Treatment Research to Improve Cognition in Schizophrenia

MCCB: MATRICS Consensus Cognitive Battery

MEG: magnetoencephalography

mGluR: metabotropic glutamate receptor

MMSE: Mini Mental State Exam

mPFC: medial prefrontal cortex

MPQ: McGill Pain Questionnaire

MPI-PS: multidimensional pain inventory pain severity scale
LIST OF ABBREVIATIONS

MRI: magnetic resonance imaging
MWM: Morris water maze
n: sample size
NA: noradrenaline
N/A: not applicable
ND: not detected in sample
NE: northeast
N-methyl-5-HT: N-methyl-5-hydroxytryptamine
NMDA: N-methyl-D-aspartic acid
NOR: novel object recognition
NRS: numerical rating scales
NSAID: non-steroidal anti-inflammatory drug
NW: northwest
p: statistical significance
PAG: periaqueductal gray
PASAT: Paced Auditory Serial Addition Task
PB: phosphate buffer; parabrachial nuclei
PBS: phosphate-buffered saline
PCC: posterior cingulate cortex
PET: positron emission tomography
PFC/PF: prefrontal cortex
PHQ-9: 9-item Patient Health Questionnaire
PHQ: Patient Health Questionnaire
PPC: posterior parietal cortex
PPI: present pain intensity
PRIME: Prevalence, Impact and Cost of Chronic Pain
PSL: partial sciatic ligation
R²: correlation coefficient (squared)
RBANS: Repeatable Battery for the Assessment of Neuropsychological Status
rpb: point-biserial correlation coefficient
SAI: State Anxiety Inventory
SD: standard deviation
SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS: sodium dodecyl sulphate
SE: southeast
SEM: standard error of the mean
SF-36: Medical Outcomes – Short Form
SF-MPQ: short-form MPQ
SMA: supplementary motor area
SNI: spared nerve injury
SNK: Student-Newman-Keuls
SNL: spinal nerve ligation
SPECT: single-photon emission computed tomography
SPSS: Statistical Package for the Social Sciences
STAI: State-Trait Anxiety Inventory
STZ: streptozotocin
SW: southwest
SYP: synaptophysin
TBS: tris-buffered saline
TCA: tricyclic antidepressant
TEA: Test of Everyday Attention
TEMED: tetramethylethylenediamine
THC: $\Delta^9$-tetrahydrocannabinol
TNF-α: tumour necrosis factor α
TRPV1: transient receptor potential vanilloid 1
VAS: visual analogue scales
VIF: variance inflation factor
w: width
WAD: whiplash-associated disorder
WAIS-III: Wechsler Adult Intelligence Scale-III
WAIS-R: Wechsler Adult Intelligence Scale – Revised
WCST: Wisconsin Card Sorting Test
WCST–CV4: Wisconsin Card Sorting Test – Computerised Version 4
WHYMPI: West Haven-Yale Multidimensional Pain Inventory
WMS-III: Wechsler Memory Scale-III
WMS-R: Wechsler Memory Scale – Revised
VAS: visual analogue scale
List of Publications

Publications in Refereed Journals


Published Conference Proceedings


Unpublished Conference Proceedings


Geoghegan, E., Moriarty, O., Finn, D. P. and Roche, M. (2010). Enhanced IL1β expression in the prefrontal cortex is associated with altered nociceptive responding in an animal model of depression. 7th Forum of European Neuroscience (Federation of European Neurosciences Societies), Amsterdam, The Netherlands, July 2010.


Chapter 1 General Introduction

Pain is a subjective, multidimensional experience that can have a marked impact on both the physiological and psychological state of an individual. The International Association for the Study of Pain (IASP) defines pain as “an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (IASP Task Force on Taxonomy, 1994). This definition is based on the concept of pain as a perception rather than as a purely sensory modality, and takes into account the fact that for pain to be consciously experienced, cognitive processing is required. Melzack and Casey’s (1968) division of pain into sensory-discriminative, motivational-affective and cognitive-evaluative dimensions also supports this concept. Chronic pain (defined as pain persisting for 3–6 months or longer) generally exceeds the duration of the precipitating noxious stimulus or injury, and may be neuropathic, inflammatory or idiopathic in nature (Aguggia, 2003). Epidemiological studies have indicated the extent of the problem of chronic pain in the general population. A cross-national, multicentre study by the World Health Organisation found that the average prevalence of persistent pain was 22% in the primary healthcare system (Gureje et al., 1998), while a survey of chronic pain in Europe estimated that 19% of adults suffer from chronic pain of moderate to severe intensity (Breivik et al., 2006). Of particular relevance to the Irish healthcare system, the recent PRIME (Prevalence, Impact and Cost of Chronic Pain) study found the prevalence of chronic pain in Ireland to be 35.5% (Raftery et al., 2011). Within this sample, approximately 12% of participants were unable to work, or were working reduced hours as a result of their pain, and those with chronic pain were more likely to suffer from depression (Raftery et al., 2011). The prevalence in this study was notably higher
than the figure of 19% reported by Breivik et al. (2006). Possible explanations for this discrepancy include differences in the “diagnostic” criteria used, a higher proportion of respondents over the age of 45 in the PRIME study, and a potential response bias. Inflammatory conditions (such as osteoarthritis and rheumatoid arthritis) and neuropathic pain account for a significant proportion of the incidence of chronic pain (Breivik et al., 2006; Torrance et al., 2006). Figure 1.1 lists a number of common causes of chronic pain identified by Breivik et al. (2006) in a large-scale survey of pain in Europe. This list is not exhaustive and does not identify some common conditions of which chronic pain is a symptom, including metabolic disorders such as diabetes, ischemic conditions, cancer, HIV/AIDS, fibromyalgia and others. A comprehensive analysis classifying chronic pain patients according to such pathological conditions does not appear to have been carried out to date.

Figure 1.1: The conditions which account for chronic pain in Europe (adapted from Breivik et al. (2006); for this study, the sample size was \( n = 4292 \)).

Chronic pain has biopsychosocial implications, affecting relationships, capacity for work, mood and quality of life (Hart et al., 2000). Chronic pain patients
report disabling limitations on physical function (van Dijk et al., 2009). Furthermore, cognitive functioning is thought to be affected in chronic pain patients. It is hypothesised that because neural systems involved in cognition and pain processing are closely linked, they may modulate one another reciprocally. Cognition has been described as the brain’s acquisition, processing, storage and retrieval of information (Lawlor, 2002). However, cognition may be considered an umbrella term that can also be used to describe integrative neuropsychological processes such as mental imaging, problem solving and perception, and is pertinent to the experience of emotion and affect. Pain and cognition share an inherent overlap owing to the fact that pain itself has a cognitive-evaluative component, requiring learning, recall of past experiences and active decision making. This should be borne in mind when assessing the effects of pain on cognitive function. A subset of cognitive domains appear to be particularly affected by chronic pain, including attention, learning and memory, speed of information processing, psychomotor ability and executive function. Loss of function in these domains is likely to impact severely on the execution of daily tasks and, therefore, negatively affect the chronic pain patient. Pain is also intrinsically related to other psychological factors such as mood, stress and anxiety, as well as parameters such as coping, disability and quality of life. A detailed evaluation of the interaction of pain with such factors is beyond the scope of this thesis, but may be found elsewhere (Gureje, 2008; López-López et al., 2008; Gustin et al., 2011; Breivik et al., 2006; Campbell et al., 2003; Dersh et al., 2002; Hart et al., 2003; Jensen et al., 2007; McCracken and Eccleston, 2003; Muñoz and Esteve, 2005; Pincus et al., 2007; Vowles and McCracken, 2008). Studies that examine the analgesic effects of cognitive interventions (for example, distraction, hypnosis and mindfulness) have also been omitted, except where the converse effects of pain on cognition have also been examined, or where the results provide insights into potential mechanisms of pain-induced cognitive impairment. In this chapter, the clinical studies which have directly measured cognitive function in chronic pain patients will be
reviewed, as will the available literature on cognitive performance in animal models of pain, to establish the current status of research on the link between chronic pain and cognition. The neural substrates which may be responsible for impaired cognition during the experience of chronic pain will also be examined. These include the neuroanatomical structures, neurotransmitter systems and other neuromodulators common to pain and cognition. The possible confounding effects of pharmacological analgesic treatment on cognition are also considered.

1.1 Evidence for pain-related cognitive impairment

1.1.1 Clinical studies

Disruption of cognitive processing has been investigated in a variety of common chronic pain syndromes, with studies focusing on a number of different types of cognitive output. The methodology employed in these studies is most commonly a test battery, which may typically include pain questionnaires and numerical rating scales (NRS) and/or visual analogue scales (VAS) to measure pain, coupled with tests of cognition. Cognitive function may be assessed using subjective self-report measures or objectively with formal, empirically validated neuropsychological tests (Ersek et al., 2004). Comorbid affective disorders (such as depression and anxiety), and the effects of sleep disturbance and medication use are sometimes, but not always, considered and they present an interesting dichotomy in experimental approach. While some researchers may consider these factors integral to the complex experience of chronic pain, others may wish to minimise their confounding effects and choose to exclude patients from research studies based on these factors. Table 1.1 has been generated following an in-depth review of the literature and gives a comprehensive summary of studies which have objectively investigated altered cognitive function in patients with chronic pain. The majority of the cognitive parameters investigated relate to attention, learning and memory, speed of processing, psychomotor ability and executive function.
1.1.1.1 Attention

Due to its biological salience, pain is an inherently attention-demanding sensory process. Hence, the effects of pain on attention are particularly well studied. Chronic pain patients frequently self-report difficulty with attention (Dufton, 1989; Jamison et al., 1988; Kewman et al., 1991; McCracken and Iverson, 2001; Muñoz and Esteve, 2005), and empirical studies have also demonstrated attentional deficits in chronic pain patients (Alanoglu et al., 2005; Bosma and Kessels, 2002; Dick et al., 2002; Dick and Rashiq, 2007; Eccleston, 1994; Grace et al., 1999; Grisart and Plaghki, 1999; Oosterman et al., 2011; Ryan et al., 1993; Veldhuijzen et al., 2006a; Miró et al., 2011; Jongsma et al., 2011) (see Table 1.1). Deficits in performance were particularly apparent on attention-switching and attentional-interference tasks. It is hypothesised that pain competes with other attention-demanding stimuli for limited cognitive resources (Eccleston and Crombez, 1999; Grisart and Van der Linden, 2001). Ongoing pain stimuli may impair top-down attentional control mechanisms which filter out task-irrelevant stimuli resulting in impaired task performance (Legrain et al., 2009a). This theory may represent one possible mechanism for cognitive impairment associated with chronic pain. Deficits in attention have been demonstrated in a wide variety of chronic pain disorders including fibromyalgia (FM, a disorder of unknown aetiology, characterized by chronic musculoskeletal pain and tactile allodynia), diabetic neuropathy, chronic lower-back pain, whiplash-associated disorder (WAD), as well as in heterogeneous groups of chronic pain patients Table 1.1. Due to differences in the methodologies and inclusion criteria used in these studies, it is difficult to reach a consensus on the relative importance of different pain conditions (see also Section 1.1.3). Studies specifically assessing migraine patients generally fail to demonstrate pain-related deficits in attention Table 1.1. This may be due to decreased pain-related interference between migraine attacks.
<table>
<thead>
<tr>
<th>Cognitive variable affected</th>
<th>Cognitive tests sensitive to impaired performance</th>
<th>Type of chronic pain</th>
<th>Pain assessment method</th>
<th>Correlation between pain and cognitive performance</th>
<th>Other parameters investigated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attention</td>
<td>Numerical interference</td>
<td>Chronic intractable benign pain of lower back, limb or other $n = 22$</td>
<td>VAS, PPI of MPQ, NRS, pain intensity descriptor scale</td>
<td>No significant correlation between numerical interference task performance and pain scores</td>
<td>No association between age, pain chronicity, gender, anxiety, depression, medication or site of pain and cognitive performance. Cognition not affected in low pain subgroup</td>
<td>Eccleston (1994)</td>
</tr>
<tr>
<td>Attention</td>
<td>Stroop task</td>
<td>Chronic intractable benign pain of lower back and other $n = 33$</td>
<td>VAS</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>No association between trait-anxiety or depression and cognition, no association between pain and response accuracy</td>
<td>Grisart and Plaghki (1999)</td>
</tr>
<tr>
<td>Attention</td>
<td>TEA</td>
<td>Fibromyalgia, rheumatoid arthritis and musculoskeletal pain $n = 60$</td>
<td>VAS</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>Age, depression, anxiety, somatic awareness and catastrophizing were accounted for statistically. Opioid use did not affect TEA performance</td>
<td>Dick et al. (2002)</td>
</tr>
<tr>
<td>Attention</td>
<td>Probe task</td>
<td>Chronic low back pain, failed back surgery syndrome, radiculopathy, pain in lower limbs, neuropathy, painful scarring</td>
<td>VAS</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>Chronic pain patients had significantly lower IQ scores than controls on adapted National Adult Reading Test</td>
<td>Veldhuijzen et al. (2006a)</td>
</tr>
<tr>
<td>Attention, reaction time</td>
<td>Attentional network test-interactions (ANT-I)</td>
<td>Fibromyalgia $n = 33$</td>
<td>ACR diagnostic criteria, MPQ</td>
<td>No significant correlations between attention task performance and pain scores</td>
<td>Female only cohort. Reaction time also correlated with anxiety and depression scores and sleep quality was predictor of attentional alertness</td>
<td>Miró et al. (2011)</td>
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</tbody>
</table>

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<thead>
<tr>
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<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attention, reaction time</td>
<td>Integneuro test battery (tasks analogous to Stroop, Go-NoGo, spatial span, reaction time tasks)</td>
<td>Chronic pain associated with chronic pancreatitis n = 16</td>
<td>Diagnosis of chronic pancreatitis with associated chronic pain</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>Verbal memory, executive function and alternative psychomotor and attention tasks were not affected by pain. Pain duration was a significant predictor of cognitive impairments</td>
<td>Jongsma et al. (2011)</td>
</tr>
<tr>
<td>Attention, maintenance of a working memory trace</td>
<td>TEA, spatial span test (mirror task)</td>
<td>Chronic pain of joints back, limbs or other n = 24</td>
<td>NRS, MPQ</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>A subset of patients showed no impairment on TEA, no effect of pain on spatial span orientation test or reading span test</td>
<td>Dick and Rashiq (2007)</td>
</tr>
<tr>
<td>Attention, working memory, immediate and delayed verbal memory, broad cognitive functioning</td>
<td>Boundon-Vos test, Digit-span backward test, Story recall subtest of the Rivermead Behavioural Memory Test, MMSE</td>
<td>Chronic visceral, musculoskeletal or neuropathic pain or other (e.g., migraine)</td>
<td>VAS</td>
<td>Significant correlation between working memory performance and pain intensity ($r = -0.38, p &lt; 0.05$)</td>
<td>No association between pain intensity and semantic memory, immediate or delayed verbal memory, or visuospatial memory. Sleep quality and depression did not correlate with cognitive performance. Opioid use and recruitment procedure did not affect task performance. Correlation was significant after adjusting for age</td>
<td>Oosterman et al. (2011)</td>
</tr>
<tr>
<td>Immediate verbal memory, delayed memory and sustained concentration</td>
<td>WMS-R, PASAT</td>
<td>Fibromyalgia n = 30</td>
<td>MPI-PS</td>
<td>Significant correlation between WMS-R general memory ($r = -0.35, p &lt; 0.05$) and PASAT scores ($r = -0.36, p &lt; 0.05$) and MPI-PS pain scores</td>
<td>No association between pain and auditory verbal learning, visual memory. Anxiety and self-reported cognitive complaints were correlated with cognitive performance</td>
<td>Grace et al. (1999)</td>
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<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>Working memory capacity, free recall, and recognition memory</td>
<td>Computer based reading span test, word list recall and recognition tests</td>
<td>Fibromyalgia</td>
<td>MPQ, AIMS pain subscale</td>
<td>Significant correlation between working memory capacity ($r = -0.466$, $p = 0.022$), free recall ($r = -0.607, p = 0.002$) and recognition memory ($r = -0.555, p = 0.005$) and AIMS pain scores. Significant correlation between free recall ($r = -0.441, p = 0.031$) and MPQ scores.</td>
<td>Female subject cohort. No association between pain and information-processing speed or verbal fluency. No association between depression or anxiety and cognitive performance. Self-reported cognitive complaints were correlated with cognitive task performance.</td>
<td>Park et al. (2001)</td>
</tr>
<tr>
<td>Immediate and delayed spatial memory</td>
<td>Rey Complex Figure test (Korean version)</td>
<td>Fibromyalgia</td>
<td>ACR diagnostic criteria, FIQ (Korean version), number of tender points</td>
<td>No significant correlation between pain measures and spatial memory.</td>
<td>Female only cohort. Delayed verbal recall and spatial reversal impaired in fibromyalgia group but not significant when depression included as a covariate. Spatial memory correlated with CPT performance suggesting dependence on attention.</td>
<td>Kim et al. (2011)</td>
</tr>
<tr>
<td>Spatial working and long-term memory</td>
<td>Corsi Block span test, Rey Visual Design Learning test</td>
<td>Fibromyalgia</td>
<td>MPQ, pain experience scale</td>
<td>No significant correlation between Corsi Block span test and Rey Visual Design Learning test performance and pain scores.</td>
<td>No association between pain and verbal working or long-term memory, or attention. No association between depression and cognitive performance.</td>
<td>Luerding et al. (2008)</td>
</tr>
<tr>
<td>Verbal memory</td>
<td>Digit span forward</td>
<td>Chronic lower back pain</td>
<td>MPQ-SF</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>Older subject cohort. No association between pain and attention and attention mental flexibility.</td>
<td>Buckalew et al. (2008)</td>
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<thead>
<tr>
<th>Cognitive variable affected</th>
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<tbody>
<tr>
<td>Verbal memory, speed of information processing and mental flexibility</td>
<td>12-word test, digit span forward and reverse, digit-symbol coding, sequential number-letter combination test</td>
<td>Chronic spinal pain</td>
<td>VAS, MPQ</td>
<td>Significant correlation between 12-word test and number-letter combination scores and VAS and MPQ subscales ($p &lt; 0.05$, $r$ values not quoted)</td>
<td>No effect on pain on tasks of attention executive function or Rey memory tests. Anxiety and catastrophizing also correlated with task performance. Test subjects split into older and younger groups, no significant differences between groups.</td>
<td>Melkumova et al. (2011)</td>
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<tr>
<td>Psychomotor efficiency</td>
<td>Grooved pegboard test, Digit vigilance test, Embedded figures test, WAIS-R digit symbol test</td>
<td>Diabetic neuropathy</td>
<td>Clinical examination</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>No association between pain and spatial processing, verbal intelligence, learning, memory, problem solving, or simple motor speed</td>
<td>Ryan et al. (1992)</td>
</tr>
<tr>
<td>Psychomotor efficiency, sustained attention, visual scanning and decision making, mental flexibility</td>
<td>Grooved pegboard test, Digit Vigilance Test, Embedded Figures Test, Trail Making Test</td>
<td>Diabetic neuropathy</td>
<td>Clinical examination</td>
<td>No correlation analysis performed for cognition and pain ratings but diagnosis of neuropathy was correlated with impaired performance on Digit Vigilance Test ($r^2 = 0.062$, $p &lt; 0.05$), Embedded Figures Test ($r^2 = 0.046$, $p &lt; 0.05$), Grooved Pegboard test ($r^2 = 0.087$, $p &lt; 0.05$) and Trail Making test ($r^2 = 0.027$, $p &lt; 0.05$)</td>
<td>No association between neuropathy and memory</td>
<td>Ryan et al. (1993)</td>
</tr>
<tr>
<td>Speed of information processing, attention</td>
<td>P300 latency and amplitude</td>
<td>Fibromyalgia</td>
<td>ACR diagnostic criteria, SF-36 pain subscale</td>
<td>No significant correlation observed between SF-36 pain subscale and the P300 latency or amplitude</td>
<td>Female subject cohort. No correlation between disease duration, tender point count or SF-36 subscales and P300</td>
<td>Alanoglu et al. (2005)</td>
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<td>Cognitive variable affected</td>
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<tr>
<td>Psychomotor efficiency</td>
<td>Grooved pegboard test</td>
<td>Diabetic neuropathy</td>
<td>Clinical examination</td>
<td>No correlation analysis performed for cognition and pain ratings but diagnosis of neuropathy was correlated with performance on the Grooved Pegboard test <em>(r and p values not quoted)</em></td>
<td>Retinopathy, vascular disease, nephropathy, coronary artery disease systolic blood pressure and duration of diabetes also affected psychomotor efficiency, no association between pain and problem solving, learning and memory and verbal working memory</td>
<td>Ryan (2005)</td>
</tr>
<tr>
<td>Psychomotor efficiency, broad cognitive functioning, immediate and delayed memory, language and metal flexibility</td>
<td>Grooved pegboard test, MMSE, RBANS immediate and delayed memory subtests, Trail Making Test</td>
<td>Chronic Low Back Pain <em>n = 163</em></td>
<td>MPQ-SF</td>
<td>Significant correlation between neuropsychological test scores and MPQ scores <em>(r = −0.17, p &lt; 0.001)</em>, most strongly between mental flexibility and psychomotor efficiency</td>
<td>Older adult patient cohort. Attention and visuospatial memory were negatively correlated with pain scores but were not significantly impaired in chronic pain patients compared with controls. Depression and presence of comorbidities were not correlated with cognitive performance</td>
<td>Weiner et al. (2006)</td>
</tr>
<tr>
<td>Psychomotor efficiency, working memory</td>
<td>WAIS-III digit-symbol test</td>
<td>Undiagnosed chronic widespread pain</td>
<td>ACR diagnostic criteria, number of pain sites</td>
<td>Significant correlation between digit-symbol test performance and pain status <em>(p = 0.04)</em>, and between digit-symbol test performance and number of pain sites <em>(p = 0.048, r values not quoted)</em></td>
<td>No effect of pain on visual memory, visual constructional ability or recognition memory. Age, test centre, depression, number of co-morbidities and current smoking affected cognitive performance, but correlation was significant after adjusting for these factors</td>
<td>Lee et al. (2010)</td>
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</tr>
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<tbody>
<tr>
<td>Reaction time</td>
<td>Visual reaction time task</td>
<td>Migraine (interictal)(n = 60)</td>
<td>Diagnosis of migraine according to IHS criteria</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>No effect of pain on attention, verbal and visual memory, perceptual-motor coordination, visual perception or abstract reasoning. No effect of anxiety on cognition. Duration of illness and number of migraine attacks per month correlated with cognitive performance</td>
<td>Calandre et al. (2002)</td>
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<td>Reaction time and working memory</td>
<td>Verbal and spatial reaction time and working memory tasks (n = 30)</td>
<td>Chronic whiplash-associated disorder</td>
<td>VAS</td>
<td>Significant correlation between verbal reaction time and VAS score (rated after task) ((r &gt; -0.80, p &lt; 0.05))</td>
<td>Medication discontinued for 16hrs before testing</td>
<td>Antepohl et al. (2003)</td>
</tr>
<tr>
<td>Reaction time, psychomotor speed</td>
<td>Continuous reaction time test, finger tapping test (n = 155)</td>
<td>Chronic non-malignant pain</td>
<td>VAS</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>No association between pain and PASAT or MMSE performance. Age, gender, education, sedation and type of medication also influenced cognitive performance</td>
<td>Sjögren et al. (2005)</td>
</tr>
<tr>
<td>Reaction time, and visuospatial awareness</td>
<td>Performance assessment battery (n = 20) (Manikin test, psychomotor vigilance and 4CRT test)</td>
<td>Persistent spinal pain</td>
<td>MPQ</td>
<td>Significant correlation between Manikin test accuracy and MPQ pain rating index score ((r = -0.301, p = 0.019))</td>
<td>No association between pain anxiety on cognitive performance</td>
<td>Haman and Ruyak (2005)</td>
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<table>
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<tr>
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<td>Perceptual learning ability</td>
<td>2-point discrimination test</td>
<td>Complex regional pain syndrome</td>
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<td>$n = 12$</td>
<td>Medical interview</td>
<td>Significant correlation between two-point discrimination thresholds on affected side and MPQ pain rating index score</td>
<td>Edema and stimulus evoked pain did not affect two-point discrimination thresholds</td>
<td>Maihöfner and DeCol (2007)</td>
</tr>
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<td>Executive function</td>
<td>Process dissociation procedure applied to a cued recall task</td>
<td>Chronic musculoskeletal pain and various peripheral neuropathic pain syndromes</td>
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<td></td>
<td></td>
<td>$n = 18$</td>
<td>NRS</td>
<td>No correlation analysis of task performance and pain rating performed</td>
<td>Pain catastrophizing, kinesophobia, pain anxiety symptoms and anxiety scores were negatively correlated with cognitive performance</td>
<td>Grisart and Van der Linden (2001)</td>
</tr>
<tr>
<td>Divided and selective attention, verbal learning and memory and spatial memory</td>
<td>Stroop task, PASAT, CVLT, Rey-Osterrieth Complex Figure Test</td>
<td>Chronic whiplash-associated disorder</td>
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<td></td>
<td>$n = 31$</td>
<td>Quebec task force on Whiplash-associated Disorder classification</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>Whiplash patients had high hypochondriasis and hysteria scores</td>
<td>Bosma and Kessels (2002)</td>
</tr>
<tr>
<td>Mental flexibility</td>
<td>D-KEFS Trails Number-Letter Switching test</td>
<td>Chronic lower back pain, osteoarthritis, fibromyalgia, peripheral neuropathy, myofascial pain, osteoporosis, spinal stenosis, headache, gout, vulvodynia, carpal tunnel syndrome, costochondritis, oesophagitis, post-herpetic neuralgia, rheumatoid arthritis, trigeminal neuralgia</td>
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<td>$n = 56$</td>
<td>SF-MIQ</td>
<td>No significant correlation between D-KEFS Trails Number-Letter Switching and SF-MIQ rating ($p = 0.056$)</td>
<td>Older adult subject cohort. No effect of pain on MMSE score, free or paired recall, or psychomotor speed. No effect of depression, opioid use, sleep disturbances, Cumulative Illness Rating Scale score or education on cognition</td>
<td>Karp et al. (2006)</td>
</tr>
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<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cognitive flexibility/executive function, problem solving</td>
<td>Tower of Hanoi-3, Object alternation test</td>
<td>Chronic migraine ( n = 23 )</td>
<td>Diagnosis of migraine according to IHS criteria, episodes occurring 15 or more days per month</td>
<td>No correlation analysis of cognitive variables with pain performed</td>
<td>Female only cohort. Cognitive flexibility tended to correlate with hypomania but not with other personality measures, depression or anxiety. Pain did not affect emotional decision making.</td>
<td>Mongini et al. (2005)</td>
</tr>
<tr>
<td>Executive function and emotional decision making</td>
<td>IGT, CALT</td>
<td>Fibromyalgia ( n = 15 )</td>
<td>Diagnosis of fibromyalgia</td>
<td>No correlation analysis of cognitive variables with pain performed</td>
<td>No effect of pain on attention or general cognitive function (Stroop task or WAIS sub-scales). CALT and IGT performance correlated with mood. Effect of pain on IGT was still present after controlling for mood.</td>
<td>Walteros et al. (2011)</td>
</tr>
<tr>
<td>Executive function and emotional decision making</td>
<td>WCST, IGT</td>
<td>Fibromyalgia ( n = 36 )</td>
<td>WHYMPI</td>
<td>Significant correlation between WCST ( r = -0.23, p = 0.03 ) and IGT ( r = -0.25, p = 0.02 ) and pain intensity scores. Significant correlation between WCST ( r = -0.25, p = 0.02 ) and IGT ( r = -0.25, p = 0.02 ) and pain interference scores</td>
<td>Female subject cohort. Pain patients showed significantly greater harm avoidance but this did not affect cognition. Years since fibromyalgia diagnosis and duration of pharmacological treatment were correlated with IGT performance</td>
<td>Verdejo-Garcia et al. (2009)</td>
</tr>
<tr>
<td>Emotional decision making</td>
<td>IGT</td>
<td>CRPS, chronic back pain ( n = 38 )</td>
<td>SF-MPQ</td>
<td>Significant correlation between SF-MPQ rating in chronic back pain group only ( r = -0.75, p &lt; 0.003 )</td>
<td>Pain did not affect WCST performance, attention, short-term memory or general intelligence. Correlation was significant after adjusting for age and pain chronicity</td>
<td>Apkarian et al. (2004a)</td>
</tr>
</tbody>
</table>

Continued on next page ....
<table>
<thead>
<tr>
<th>Cognitive variable affected</th>
<th>Cognitive tests sensitive to impaired performance</th>
<th>Type of chronic pain</th>
<th>Pain assessment method</th>
<th>Correlation between pain and cognitive performance</th>
<th>Other parameters investigated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broad cognitive functioning</td>
<td>MMSE, CCSE</td>
<td>Migraine, cluster headache and chronic daily headache (during headache intervals)</td>
<td>Diagnosis of migraine and cluster headache according to IHS criteria, chronic daily headache met migraine criteria for individual headaches and incidence exceeded 15 days per month</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>No effect of depression on cognition. MMSE and CCSE rating decreased only during headache intervals (within subjects design). Gender and age significantly affected cognitive performance</td>
<td>Meyer et al. (2000)</td>
</tr>
<tr>
<td>Broad cognitive functioning</td>
<td>MMSE</td>
<td>Diabetic neuropathy, trigeminal neuralgia, CRPS, post-herpetic neuralgia, entrapment syndromes, post-stroke pain, peripheral neuropathies and neuralgias, radiculopathy, lumbar pain, slipped disc, spinal canal stenosis, spondylolysis, spondylothesis, surgical trauma, musculoskeletal pain and rheumatologic causes</td>
<td>VAS, SF-MPQ</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>Age, anxiety, depression and obesity also affected cognition</td>
<td>Povedano et al. (2007)</td>
</tr>
</tbody>
</table>
Table 1.1 (continued from previous page)

<table>
<thead>
<tr>
<th>Cognitive variable affected</th>
<th>Cognitive tests sensitive to impaired performance</th>
<th>Type of chronic pain</th>
<th>Pain assessment method</th>
<th>Correlation between pain and cognitive performance</th>
<th>Other parameters investigated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broad cognitive functioning</td>
<td>MMSE</td>
<td>Fibromyalgia</td>
<td>SF-MPQ</td>
<td>No correlation analysis performed for cognition and pain ratings</td>
<td>Age, pain severity and presence of anxiety and depression did not affect frequency of cognitive impairment but anxiety and depression scores were higher in fibromyalgia patients with impaired cognition</td>
<td>Rodríguez-Andreu et al. (2009)</td>
</tr>
</tbody>
</table>

**Abbreviations:** 4CRT: four-choice reaction time; ACR: American College of Rheumatology; AIMS: Arthritis Impact Measurement Scales; CALT: Conditioned associative learning task; CCSE: Cognitive Capacity Screening Examination; CRPS: Complex Regional Pain Syndrome; CVLT: California Verbal Learning Test; D-KEFS: Delis-Kaplan Executive Function System; FIQ: Fibromyalgia impact questionnaire; IGT: Iowa Gambling Task; IHS: International Headache Society; MPQ: McGill Pain questionnaire; MPI-PS: multidimensional pain inventory pain severity scale; MMSE: Mini Mental State Exam; n: sample size; NRS: numerical rating scale; PPI: present pain intensity; PASAT: Paced Auditory Serial Addition Task; SF-36: Medical Outcomes – short form; RBANS: Repeatable Battery for the Assessment of Neuropsychological Status; SF-MPQ: short-form MPQ; TEA: Test of Everyday Attention; WAIS-R: Wechsler Adult Intelligence Scale-Revised; WCST: Wisconsin Card Sorting Test; WHYMPI: West Haven-Yale Multidimensional Pain Inventory; WMS-R: Wechsler Memory Scale – Revised; VAS: visual analogue scale.

Table 1.1: Summary of clinical studies investigating the effect of chronic pain on cognition.
1.1.1.2 Learning, memory and general cognition

Self-reported deficits in memory are common among chronic pain patients (Schnurr and MacDonald, 1995; Ling et al., 2007). Learning and memory have been evaluated psychometrically in chronic pain patients, with patients performing poorly compared with controls on parameters including spatial (Dick and Rashiq, 2007; Luerding et al., 2008; Kim et al., 2011) and verbal (Antepohl et al., 2003; Bosma and Kessels, 2002; Grace et al., 1999; Oosterman et al., 2011; Park et al., 2001; Weiner et al., 2006; Buckalew et al., 2008; Melkumova et al., 2011) working memory capacity and recall, recognition memory (Park et al., 2001), and long-term spatial memory (Luerding et al., 2008). Memory impairments have been shown in a number of diseases of which chronic pain is a symptom, but are particularly prevalent in FM (Table 1.1; see also Glass, 2009, for a review). Studies of memory in chronic pain patients have largely tested parameters of working (explicit) memory. Implicit (semantic, procedural, and conditioned) memory is generally considered an automated process, less likely to be affected by the presence of chronic pain (Grisart and Van der Linden, 2001). However, FM patients have also shown deficits in semantic memory (verbal knowledge) tasks where they were required to select the antonym or synonym of a presented word (Park et al., 2001). Fatigue and depression are also common in FM, however, and may affect performance in these cognitive domains.

Aversive memory may be of particular interest in examining the relationship between chronic pain and cognitive function, due to the shared neuroanatomical regions and neural processes involved. However, the effects of chronic pain on this specific subtype of memory have been less well studied. This issue will be addressed further in Chapter 4.

Chronic pain patients also perform poorly compared with controls on general screening measures of cognition, such as the Mini Mental State Exam (MMSE) (Meyer et al., 2000; Oosterman et al., 2011; Weiner et al., 2006), Cognitive Capacity Screening Exam (CCSE) (Meyer et al., 2000), and electroencephalographic
measurement of the P300 event-related potential (Alanoglu et al., 2005). Furthermore, the prevalence of clinically relevant cognitive impairment (measured as an MMSE score \( \leq 24 \)) was higher in chronic pain patients than in the general population (Povedano et al., 2007; Rodríguez-Andreu et al., 2009). As is the case for attentional tasks, deficits were observed across a variety of chronic pain conditions (Table 1.1). FM patients achieved significantly lower scores on the MMSE than patients with a diagnosis of neuropathic or mixed pain (Rodríguez-Andreu et al., 2009). In that study, there did not appear to be a direct link between impaired performance and pain severity. An inverse relationship between MMSE or CCSE performance and pain scores in chronic pain patients has not been reported. However, other studies have shown an inverse correlation between pain scores and cognitive performance (see Table 1.1).

1.1.1.3 Speed of information processing and psychomotor ability

Chronic pain patients display longer reaction times than matched controls in a variety of standardised cognitive tests (Alanoglu et al., 2005; Antepohl et al., 2003; Biessels et al., 2007; Calandre et al., 2002; Harman and Ruyak, 2005; Sjøgren et al., 2005; Miró et al., 2011; Jongsma et al., 2011), in particular on tests related to psychomotor ability (Harman and Ruyak, 2005; Lee et al., 2010; Ryan, 2005; Ryan et al., 1992, 1993). Impaired perceptual learning ability has also been shown in chronic pain patients (Maihöfner and DeCol, 2007). These results suggest a common pattern of impaired perceptual-motor coordination in chronic pain (Table 1.1). The studies in Table 1.1 appear to suggest a high prevalence of psychomotor dysfunction in diabetic neuropathy; however, it should be noted that this parameter has not been routinely tested in other chronic pain disorders.

1.1.1.4 Executive function

Executive function loosely defines neurological processes that enable more complex cognitive tasks such as planning, organisation, control of conflicting thoughts,
goal-directed behaviour, initiation of action and assessing the consequences of actions. Clinically, executive function is assessed using interference tasks such as the Wisconsin Card Sorting Test (Grant and Berg, 1948) or similar conditioned associative learning tasks (Petrides et al., 1993), the Process Dissociation Procedure (Jacoby, 1991), object alternation tasks (Freedman and Oscar-Berman, 1986; Freedman, 1990) and flexibility tasks such as the Trail Making Test Part B (Adju tant General’s Office, 1944), and letter-number sequencing (Abeare et al., 2010). Patient performances on these tests appear to show that controlled executive-type functions are affected by chronic pain (Karp et al., 2006; Ryan et al., 1993; Verdejo-García et al., 2009; Weiner et al., 2006; Mongini et al., 2005; Walteros et al., 2011; Melkumova et al., 2011) and may be more severely affected than less complex, automatic processes (for example, fixed sequences of operations that do not require higher control) (Grisart and Van der Linden, 2001) (Table 1.1). Complex tests of attention, such as those that involve interference or attention switching, may also require executive function, and chronic pain patients perform poorly on such tests (Bosma and Kessels, 2002; Eccleston, 1994; Karp et al., 2006; Ryan et al., 1993; Miró et al., 2011; Jongsma et al., 2011). For example, Eccleston (1994) showed that pain affected performance on an attentional-interference task, but only when the task was at its most complex. Studies by Suhr (2003) and Scherder et al. (2008), however, failed to show any executive function deficits in chronic pain patients. A study by Oosterman and colleagues (2009) actually found a positive relationship between self-reported pain and executive function (increased pain levels associated with improved cognitive function). However, this study did not include a pain-free control group and was carried out specifically in elderly pain patients (ages 84.6 ± 0.5 years). Emotional decision making and emotional self-regulation are also thought to involve higher executive functioning and appear to be compromised in some chronic pain patients (Apkarian et al., 2004a; Solberg Nes et al., 2009; Verdejo-García et al., 2009). Interestingly, a study by Veldhuijzen et al. (2006a) also found that chronic pain patients, while
CHAPTER 1. GENERAL INTRODUCTION

showing increased errors in an attentional task, had shorter response times than healthy controls. Together, these findings indicate that chronic pain may be associated with increased impulsivity or impaired attentional control. Executive function is under control of frontal brain regions that are also involved in pain processing. The potential forebrain mechanisms by which pain may affect executive function are discussed in Section 1.2. Deficits in executive function have been shown in a number of chronic pain disorders, and do not appear to follow a disease-specific pattern (Table 1.1). However, emotional decision making was found to be impaired in patients with chronic back pain but not in patients suffering from complex regional pain syndrome (Apkarian et al., 2004a).

1.1.2 Preclinical studies

Preclinical studies provide an opportunity to further explore the mechanisms mediating pain-related impairment of cognition. However, this type of basic research has been under-utilised, with relatively few published studies attempting to model the clinical phenomenon of pain-related cognitive impairment in laboratory animals, despite the availability of well-validated animal models of pain and cognitive impairment. Some commonly used models of chronic pain, both inflammatory and neuropathic, are listed in Table 1.2. These models differ in the type and site of the injury, the sensory symptoms expressed, and their utility in cognitive research.

There is some evidence that pain impacts negatively on cognition in rodents (see Table 1.3). Studies by Cain et al. (1997) and Lindner et al. (1999) found that complete Freund’s adjuvant (CFA)-induced inflammatory pain was associated with impaired performance on a delayed nonmatching-to-position lever-press task, which is sensitive to deficits in learning, memory and attention. Millecamps et al. (2004) reported a decrease in non-selective, non-sustained attention in a rat model of visceral inflammatory pain. 2,4,6-trinitrobenzene was used to induce colitis and attentional level was assessed using a novel-object paradigm. Control rats spent a significantly larger percentage of time exploring the novel object than did colitic
<table>
<thead>
<tr>
<th>Pain Model</th>
<th>Type/position of injury</th>
<th>Type of pain modelled</th>
<th>Sensory symptoms</th>
<th>Associated confounds</th>
<th>Cognitive deficits</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA</td>
<td>Intraplantar, intradermal (base of tail) or intra-articular injection</td>
<td>Inflammatory</td>
<td>Allodynia, hyperalgesia, oedema</td>
<td>LMA deficits, illness if in systemic circulation (base of tail), decreased body weight</td>
<td>✓</td>
</tr>
<tr>
<td>Trinitrobenzene</td>
<td>Enema in colon</td>
<td>Visceral/inflammatory</td>
<td>Referred hypersensitivity</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Kaolin</td>
<td>Intra-articular injection</td>
<td>Inflammatory</td>
<td>Allodynia, hyperalgesia, oedema</td>
<td>LMA effects</td>
<td>✓</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>Intraplantar, or intra-articular injection</td>
<td>Inflammatory</td>
<td>Allodynia, hyperalgesia, oedema</td>
<td>LMA effects</td>
<td>✓</td>
</tr>
<tr>
<td>L5 transection</td>
<td>Transection of L5 spinal nerve lateral to DRG</td>
<td>Neuropathic</td>
<td>Allodynia, hyperalgesia</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>CCI</td>
<td>Loose partial ligation of the sciatic nerve</td>
<td>Neuropathic</td>
<td>Allodynia, hyperalgesia</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>SNI</td>
<td>Transection of common peroneal and tibial nerves</td>
<td>Neuropathic</td>
<td>Allodynia, hyperalgesia</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>SNL</td>
<td>Ligation of L5 and L6 spinal nerves lateral to DRG</td>
<td>Neuropathic</td>
<td>Allodynia, hyperalgesia</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>PSL</td>
<td>Partial ligation of the sciatic nerve</td>
<td>Neuropathic</td>
<td>Allodynia, hyperalgesia</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>STZ</td>
<td>Systemic injection of STZ</td>
<td>Neuropathic</td>
<td>Allodynia, hyperalgesia</td>
<td>LMA deficits, illness, decreases in body weight</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 1.2: Summary of commonly used animal models of chronic pain: sensory symptoms, potential confounds and demonstrated validity in cognitive research. Abbreviations: CCI: chronic constriction injury; CFA: complete Freund’s adjuvant; DRG: dorsal root ganglion; LMA: locomotor activity; PSL: partial sciatic ligation; SNI: spared nerve injury; SNL: spinal nerve ligation; STZ: streptozotocin.
rads. Though the paradigm was described by the authors as an attentional task, this type of object recognition task is more accurately defined as a measure of recognition memory and the ability to discriminate between novel and familiar objects. Tests such as the five-choice serial reaction-time task (5CSRTT) are regarded as more specific measures of attention. Pain-related deficits have also been demonstrated in these tasks. Animals treated with formalin showed decreased attention in a 5CSRT task (Boyette-Davis et al., 2008). Pais-Vieira and colleagues (2009a) also showed that correct responding in a similar task decreased and the number of omissions increased, following induction of chronic inflammatory pain with CFA. Work by Ford et al. (2008) also suggested an inverse relationship between selective attention towards an unfamiliar object and formalin-evoked nociceptive behaviour in rats.

Inflammatory pain has been shown to affect the performance of rats in a gambling task which is thought to be analogous to the Iowa Gambling Task and assesses emotional decision making (Zeeb et al., 2009). In two rat models of monoarthritis (intra-articular kaolin and carrageenan to the knee joint or CFA to the tibiotarsal joint of the hindpaw), pain responding was associated with a preference for a “high-risk” lever associated with larger but more infrequent rewards than the alternative lever (Ji et al., 2010; Pais-Vieira et al., 2009b). These findings parallel the clinical investigations of Verdejo-García et al. (2009) and Apkarian et al. (2004a), in which chronic pain patients showed impaired learning in the Iowa Gambling Task. However, the “emotional” processing component may be different between species.
Table 1.3: Summary of preclinical animal studies investigating the effect of pain on cognition.

<table>
<thead>
<tr>
<th>Pain model</th>
<th>Cognitive test</th>
<th>Cognitive domain</th>
<th>Key Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA-induced inflammatory pain (rat)</td>
<td>Operant delayed non-matching to position lever press task</td>
<td>Spatial learning, recognition memory and attention</td>
<td>Decrease in accuracy and decrease in number of rewards earned in pain model</td>
<td>Cain et al. (1997)</td>
</tr>
<tr>
<td>CFA-induced inflammatory pain (rat)</td>
<td>Operant delayed non-matching to position lever press task</td>
<td>Spatial learning, recognition memory and attention</td>
<td>Decrease in accuracy and decrease in response latency</td>
<td>Lindner et al. (1999)</td>
</tr>
<tr>
<td>2, 4, 6-trinitrobenzene-induced inflammatory pain (colitis, rat)</td>
<td>Novel object recognition</td>
<td>Recognition memory, attention</td>
<td>Decrease in attention towards novel object in pain model</td>
<td>Millecamps et al. (2004)</td>
</tr>
<tr>
<td>Formalin-induced inflammatory pain (rat)</td>
<td>5CSRTT (visual-stimulus nose-poke task)</td>
<td>Attention</td>
<td>Increased number of omissions in pain model</td>
<td>Boyette-Davis et al. (2008)</td>
</tr>
<tr>
<td>CFA-induced inflammatory pain (rat)</td>
<td>5CSRTT (visual-stimulus nose-poke task)</td>
<td>Attention</td>
<td>Decrease in accuracy, increased number of omissions and increase in preservative responses in pain model</td>
<td>Pais-Vieira (2009a)</td>
</tr>
<tr>
<td>Kaolin-induced inflammatory pain (rat)</td>
<td>Rodent gambling task</td>
<td>Emotional decision making</td>
<td>Increased preference for high risk level associated with larger, more infrequent rewards in pain model</td>
<td>Ji et al. (2010)</td>
</tr>
<tr>
<td>Carageenan-induced inflammatory pain (rat)</td>
<td>Rodent gambling task</td>
<td>Emotional decision making</td>
<td>Increased preference for high risk level associated with larger, more infrequent rewards and increase in number of omissions in pain model</td>
<td>Pais-Vieira et al. (2009b)</td>
</tr>
<tr>
<td>L5 transection-induced neuropathic pain (rat)</td>
<td>Morris Water maze (acquisition and probe)</td>
<td>Spatial learning and memory</td>
<td>Increased latency to platform during acquisition and decreased frequency in platform zone during probe in pain model</td>
<td>Hu et al. (2010)</td>
</tr>
<tr>
<td>SNI model of neuropathic pain (rat)</td>
<td>Morris Water maze (traditional acquisition and reversal)</td>
<td>Spatial learning and memory and cognitive flexibility</td>
<td>Decreased % of distance swam in new platform location and increased % in old location in pain model</td>
<td>Leite-Almeida et al. (2009)</td>
</tr>
<tr>
<td>SNL model of neuropathic pain (mouse)</td>
<td>Passive avoidance</td>
<td>Aversive learning</td>
<td>No impairment of passive avoidance response in pain model</td>
<td>Suzuki et al. (2007)</td>
</tr>
</tbody>
</table>

Continued on next page ....
Table 1.3: Summary of preclinical animal studies investigating the effect of pain on cognition.

<table>
<thead>
<tr>
<th>Pain model</th>
<th>Cognitive test</th>
<th>Cognitive domain</th>
<th>Key Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNI model of neuropathic pain (rat and mouse)</td>
<td>Eight-arm radial maze test</td>
<td>Spatial working memory</td>
<td>Increased re-entry into baited arms already visited in SNI rats and mice</td>
<td>Ren et al., (2011)</td>
</tr>
<tr>
<td>Partial sciatic nerve injury model of neuropathic pain (mouse)</td>
<td>Novel object recognition</td>
<td>Recognition memory, attention</td>
<td>Decrease in attention towards novel object in pain model</td>
<td>Kodama et al., (2011)</td>
</tr>
<tr>
<td>SNI model of neuropathic pain (mouse)</td>
<td>Contextual fear conditioning</td>
<td>Memory of conditioned fear</td>
<td>Inability to extinguish conditioned fear in pain model</td>
<td>Mutso et al., (2012)</td>
</tr>
</tbody>
</table>

Cognitive performance has also been investigated in animal models of chronic neuropathic pain. Streptozotocin (STZ)-induced diabetes in rats was found to be associated with impaired spatial learning in the Morris water maze (Biessels et al., 1996, 1998; Kamal et al., 2000), and type 1 insulopenic BB/Wor rats have also shown spatial memory and learning deficits (Sima and Li, 2005). It is likely that painful neuropathy was associated with these diabetic models; however, this was not directly assessed in these studies, and warrants further investigation. Abnormal sensory responses (possibly indicative of diabetic neuropathy) have been observed in other, recently developed models of diabetes (Obrosova, 2009), although cognition has not been examined extensively in these models. Recent studies by (Ren et al., 2011) and (Kodama et al., 2011) have shown deficits associated with surgical neuropathic pain models in rodents in tests of spatial working memory in the eight-arm radial maze task and novel object recognition paradigms. In the radial-arm maze, SNI rats and mice were more likely to re-enter a baited arm from which the food reward had already been collected (Ren et al., 2011). In the novel object task, mice that had undergone partial sciatic nerve injury showed less preference for the novel object than did sham controls (Kodama et al. 2011). Another recent study by Hu et al. (2010) found impairments in spatial learning...
and memory in the Morris water maze following L5 spinal nerve transection as a model of neuropathic pain in the rat. Furthermore, Leite-Almeida et al. (2009) described impaired behavioural flexibility in a rat model of neuropathic pain. In this experiment, spared nerve injury (SNI) was used to model chronic neuropathic pain. Nerve-injured rats showed impairment in the spatial reversal task of the Morris water maze. In a reversal task, rats are required to learn the position of a hidden platform according to spatial cues (as in the traditional learning task) and once the position is learned, the platform is moved to a new location. Control rats, with intact behavioural flexibility, rapidly learned the new location, while impaired, nerve-injured rats continued to explore the old location. This type of behavioural test is somewhat analogous to clinical tests of executive function, and so the preclinical models show some similarity to clinical studies that found impaired executive function in chronic pain patients. There are also studies in which neuropathic pain models were not associated with impaired cognitive function; for example, Suzuki et al. (2007) found that contextual aversive memory was not affected in mice that had undergone spinal nerve ligation (SNL) surgery as a model of neuropathic pain. Mutso et al. (2012) found that SNI rats’ ability to extinguish a conditioned fear response was impaired relative to sham rats. While this does not represent cognitive impairment as such, it is interpreted by the authors as an impairment of normal hippocampal functioning. To our knowledge, these are the first and only studies to examine, in vivo, cognitive performance in animal models of neuropathic pain, and they therefore represent an important contribution to the field.

1.1.3 Limitations of the current literature

Overall, the research published to date, both clinical and preclinical, provides a strong basis for the theory that cognitive function is impaired in chronic pain. Deficits in a number of cognitive parameters have been shown in chronic pain patients using several well-validated psychometric tests. However, there are also
studies that report no association between chronic pain and impaired cognitive function (Bell et al., 1999; Landrø et al., 1997; Pincus et al., 1998; Scherder et al., 2008; Suhr, 2003). There are a number of caveats associated with the clinical studies presented herein, including differences in screening measures used, differences in the type of chronic pain or disease state investigated and differences in accounting for control factors and comorbidities. Deficits were commonly observed in some cognitive domains but not in others, and there is no obvious pattern of common effects between studies (Table 1.1). Certain disorders, such as FM and diabetic neuropathy, appear to be consistently associated with cognitive impairments, though the spectrum of impairments differs between studies (Table 1.1). Studies which have examined cognitive performance in mixed chronic pain disorders do not provide information on whether specific impairments are more frequently observed in specific disorders. However, the investigation by Apkarian et al. (2004a) showed that pain ratings were negatively correlated with impaired performance on an emotional decision-making task (the Iowa Gambling Task) in one type of chronic pain (chronic back pain) but not in another (complex regional pain syndrome), suggesting that the type of chronic pain disorder may be important in relation to cognitive effects. In addition, pain-related cognitive impairments may be transient and reversible in some chronic pain conditions such as headache (Meyer et al., 2000). Chronic pain is frequently comorbid with affective disorders (anxiety and depression), stress and fatigue. Chronic pain has a bidirectional relationship with these conditions, through effects on neuroendocrine and neurotransmitter systems. Thus, it is possible that psychiatric symptoms or pathophysiological features that are not related to pain, but which are features of these comorbidities, may also affect the aspects of cognition highlighted above (Austin et al., 2001; Brown et al., 2002; Capuron et al., 2006; Castaneda et al., 2008; Eysenck et al., 2007; Hindmarch, 1998; Lupien et al., 2007). In addition, psychological traits such as hypervigilance and catastrophizing are common in chronic pain patients, and may affect the outcome of cognitive tests. Disease
symptoms, other than psychiatric symptoms, may also affect patients’ ability to perform well on tests of cognitive function. For example, diabetic neuropathy occurs as a result of hyperglycaemia-induced nerve damage which can also affect motor neurons. This damage may, in part, explain poor performance on tests of psychomotor ability in these patients. Certain pain conditions may also be more likely to occur in different demographic groups. For example, FM is more likely to occur in women than in men (Branco et al., 2010; White et al., 1999), and arthritis is more prevalent in older adults (Hootman et al., 2012; Frondini et al., 2007). Age and gender have been shown to influence cognitive performance (see Table 1.1), though these variables are commonly accounted for statistically using regression modelling techniques. In addition, studies examining pain-related cognitive effects have been carried out in specific demographic groups such as older adults (Karp et al., 2006; Weiner et al., 2006), female patients only (Alanoglu et al., 2005; Park et al., 2001; Verdejo-García et al., 2009) and male patients only (Lee et al., 2010). Importantly, irrespective of these additional factors, a number of studies have shown a direct correlation between the level of cognitive dysfunction and pain ratings in chronic pain patients (see Table 1.1). Furthermore, and as discussed further in Section 1.2.7 below, improvements in cognitive performance in patients suffering from chronic pain have been observed following chronic analgesic treatment (Tassain et al., 2003; Jamison et al., 2003). Together, these studies may provide the best clinical evidence for pain-related cognitive impairment.

Cognitive performance has also been investigated in experimental pain (pain induced under laboratory conditions in otherwise healthy, human subjects). A number of studies have found that pain was associated with deficits in performance (Babiloni et al., 2004; Crombez and Baeyens, 1994; Crombez et al., 1996, 1998; Legrain et al., 2009b; Moore et al., 2009; Vancleef and Peters, 2006; Walker, 1971; Buhle and Wager, 2010). Such studies can be carefully controlled and may allow strong conclusions to be drawn regarding the effects of pain \textit{per se} on cognitive functioning. However, experimental pain studies can be criticised
because of their limited ability to accurately model motivational-affective and evaluative aspects of pain (Gagliese, 2007). Furthermore, the effect of chronic pain on neuropsychological function may be cumulative, and quantitatively different from acute/experimental pain (May, 2008). Thus, the rest of this thesis will focus on chronic pain.

With regard to the preclinical animal research, the majority of studies show that pain-related cognitive impairment can be modelled in laboratory animals and demonstrate some similarities with the clinical literature. It appears that high-order executive-type functions may be most severely affected in preclinical models (Leite-Almeida et al., 2009), as is the case for clinical studies. In addition, analgesic drugs appear to reverse cognitive impairments observed in chronic pain models (Boyette-Davis et al., 2008; Cain et al., 1997; Hu et al., 2010; Lindner et al., 1999; Millecamps et al., 2004). Thus, results in preclinical models parallel those from clinical studies of pain-related cognitive impairment, suggesting that further examination of the neural mechanisms in animal models may translate to humans. However, there are again limitations associated with this type of investigation. Tests of cognition in animal models are largely based on spatial learning and memory or attention, which have also been shown to be impaired in chronic pain patients (see Table 1.1). However, in contrast to the clinical studies, assessment of cognition in animal subjects is highly dependent on locomotor activity and appetitive responding, both of which are likely to be affected in models of chronic pain. Neuropathic and inflammatory pain models were found to be associated with impaired motor function (Cain et al., 1997; Lindner et al., 1999; Leite-Almeida et al., 2009). Leite-Almeida et al. (2009) avoided the confounding effect of decreased locomotor activity in the water maze cognitive task by measuring the path length. Cain et al. (1997) and Lindner et al. (1999) did not account for the effect of impaired locomotor activity on cognitive performance, and as such, the effect of pain on task performance is difficult to interpret. Chronic pain models are also frequently associated with the expression of depressive-like
symptoms, such as increased immobility time in the forced swim test (Hu et al., 2010; Leite-Almeida et al., 2009; Suzuki et al., 2007). Anhedonia may also be a feature of animal models of chronic pain, as CFA-treated rats demonstrated a trend for decreased sucrose consumption \((p = 0.056)\) (Shi et al., 2010a). It is reasonable to assume, therefore, that motivation to earn food rewards (necessary for operant cognitive tasks), to explore (necessary for object exploration tasks), and to escape aversive situations (necessary for water maze and passive avoidance tests of cognition) may also be impaired in models of chronic pain. In the study by Millecamps et al. (2004), there was no decrease in total object exploration in colitic rats, which suggests motivation to explore was not affected in this pain model. In operant tasks, motivation is generally assessed within the test protocol by measuring the number of trials initiated by the rat (Pais-Vieira et al., 2009a) or latency to retrieve food rewards (Boyette-Davis et al., 2008). Although pain did not affect these measures, impaired motivation as a potential confound cannot be completely ruled out.

### 1.2 Potential mechanisms involved in pain-related cognitive impairment

Although the precise mechanisms have yet to be elucidated, several theories have emerged regarding the mechanisms mediating cognitive impairment in conditions of persistent pain. As discussed in the previous section, it may be argued that the cognitive impairments observed in chronic pain may simply be a consequence of the division of limited resources in discrete brain regions. The persistent nociceptive inputs associated with chronic pain may compete with other sensory inputs resulting in reduced cognitive performance (Eccleston and Crombez, 1999). Hart and colleagues (2000) proposed that neuroplastic changes occur in chronic pain and that such neural rewiring or reorganisation in the brain interferes with normal cognitive functioning. These authors also suggested that neurochemical mediators, released during chronic pain, may have a negative effect on cognitive
processing. The neuroanatomical and neurochemical substrates at which pain and
cognitive processing systems overlap (and are likely to interact with one another)
will be discussed in light of these possible mechanisms. Various tools have been
used by researchers to investigate potential mechanisms involved in pain-related
cognitive impairment. These include pharmacological manipulation, post-mortem
molecular measurements and sophisticated human imaging techniques.

1.2.1 Shared neuroanatomy in pain and cognition

Studies using neuroimaging techniques, including electroencephalography (EEG),
magnetoencephalography (MEG), functional magnetic resonance imaging (fMRI)
and positron emission tomography (PET), have greatly advanced our understand-
ing of the interaction between pain and cognitive processing at a neuroanatomical
level. The pain experience has been described as the output of an integrated
neural network or “neuromatrix” (Melzack, 1999). Somatosensory cortical areas
1 and 2 (SI and SII), the insular cortex (IC), the thalamus, the prefrontal cortex
(PFC) and the anterior cingulate cortex (ACC) have been identified as the six
brain regions most commonly activated during pain processing: see Figure 1.2
(Apkarian et al., 2005; Tracey, 2008). The periaqueductal gray (PAG), basal gan-
glia, cerebellum, amygdala and hippocampus have also, though less consistently,
shown pain-related activation.

Components of the pain neuromatrix are also critical in the processing of cog-
nitive information. The ACC is part of the medial PFC and receives input from
limbic regions such as the thalamus, the hippocampus and the amygdala (Devinski
et al., 1995; Bush et al., 2000). Its role in control of selective attention,
working memory and error awareness has been demonstrated using fMRI (Klein
et al., 2007). Buffington et al. (2005) examined activation of the ACC in healthy
subjects and chronic pain patients subjected to an acute noxious stimulus, whilst
carrying out a continuous performance task requiring sustained attention. ACC
activations were modulated by acute pain and by the sustained attention task.
The authors describe an inverse relationship between two distinct components of activation, one thought to relate to sustained attention and one to acute pain stimulation. The component related to acute pain stimulation was activated to a greater extent in chronic pain patients both before and after painful stimulation. In addition, ACC activation relating to the attentional task performance was localised differently in patients and controls. These results suggest that chronic pain alters normal processing in the ACC, which could account for deficits in cognitive function. Frankenstein et al. (2001) used fMRI to study healthy subjects undergoing the cold pressor test whilst performing a distracting verbal task. Results showed that separate areas of the ACC responded to the painful stimulus and to the distracting task. However, a subregion in the perigenual area of the ACC was co-activated in both conditions, and this finding has been replicated in other imaging studies of experimental pain in healthy subjects (Bantick et al., 2002; Valet et al., 2004). In addition, pain-evoked activity in the ACC can be altered
in healthy volunteers by hypnotic suggestions (Faymonville et al., 2000; Rainville et al., 1997), pain anxiety (Ploghaus et al., 2001) and anticipation of pain (Hsieh et al., 1999). Competition between pain and cognition for processing resources in the ACC may result in a reduced functional capacity, thereby reducing cognitive ability in pain states. The ACC has been examined in relation to pain-related memory acquisition and affective pain processing in animal models. Conditioned place-avoidance paradigms give animals free access to a dark (non-aversive) and a light (aversive) chamber. A noxious stimulus is applied when animals are in the preferred dark chamber causing the animals to move from the dark chamber to the light chamber, and to avoid the dark chamber (avoidance). This type of experiment is used to model cognitive-affective aspects of pain processing. Inflammatory pain (CFA, formalin) and neuropathic pain (SNL model) increase short-term avoidance behaviour following painful stimulation of the injured paw, compared with control animals (LaBuda and Fuchs, 2000; Pedersen and Blackburn-Munro, 2006). Lesion of rostral ACC neurons reverses formalin-induced conditioned place avoidance but does not affect nociceptive behaviours (lifting, flinching etc.), thus implicating the ACC in affective pain processing but not in sensory processing (Johansen et al., 2001). Electrical stimulation of the ACC also resulted in a reversal of avoidance behaviour in a rat model of neuropathic pain (LaBuda and Fuchs, 2005). Scopolamine, a cholinergic antagonist known to impair learning and memory, injected directly into the ACC, impairs pain-related memory acquisition in the rat (Ortega-Legaspi et al., 2003).

The IC shows somatotopic organisation (Brooks et al., 2005), is a component of the limbic system, and is activated by the experience of emotions such as happiness, sadness, anger, fear and disgust (Damasio et al., 2000; Phillips et al., 1997). The IC is interconnected with the entorhinal cortex and the amygdala (Insausti et al., 1997; Shi and Cassell, 1998) and is therefore presumed to play a role in affective processing of pain and pain control. Activation of the IC caused by experimental thermal pain was reduced when subjects were distracted by a
visual stimulus (Brooks et al., 2002). Other distracting tasks were also found to reduce pain-induced activity in the IC in healthy subjects (Bantick et al., 2002; Longe et al., 2001). Conversely, increased IC activation in pain could be associated with impaired cognitive performance, particularly in emotionally demanding tasks, such as the Iowa Gambling Task, though this hypothesis has not yet been tested.

The PAG is known to be involved in pain control and is an important component of the endogenous descending inhibitory pain pathway. Imaging studies have shown altered activity in the PAG during simultaneous cognitive and evoked-pain processing in healthy participants. Performance of a maze task (Petrovic et al., 2000) or directing attention to a painful stimulus (Tracey et al., 2002) reduced pain-induced activation in the PAG, but it is unclear whether pain may modulate cognitive function through this region.

Single-photon emission computed tomography (SPECT) has shown alterations in cerebral blood flow (hypoperfusion) in migraine patients when compared with healthy controls (Calandre et al., 2002). Anatomical areas of hypoperfusion were diffuse, but prominent in frontal and parietal regions. Altered cerebral blood flow was found to be associated with significant impairment on cognitive tasks of verbal and visual memory. In a separate study, a heterogeneous sample of chronic pain patients showed altered EEG patterns, compared to controls when carrying out a visual task and attending to irrelevant stimuli (Veldhuijzen et al., 2006a). These authors predicted that as task difficulty increased, the amplitude of P300 event-related potentials associated with task stimuli would increase and the amplitude of those associated with task-irrelevant stimuli would decrease. However, chronic pain patients did not show a decrease in response to task-irrelevant stimuli, suggesting impaired allocation of attentional resources in these patients. A further EEG study by Tiemann and colleagues (2010) in healthy subjects found that as pain-induced gamma oscillations increased, task-related visual gamma oscillations decreased. This neural response was associated with impaired task performance.
in a subset of subjects.

These studies effectively highlight the overlapping brain regions involved in pain and cognitive processes and support the “division of resource” theory for cognitive impairment in chronic pain. However, it is important to note that a number of these studies are concerned primarily with psychoanalgesic effects of cognitive interventions, and have not directly examined the negative effect of pain on cognition. In addition, the majority of these studies were performed in healthy volunteers subjected to experimental pain under laboratory conditions. Only recently have functional changes associated with pain-related cognitive impairment been directly investigated. Weissman-Fogel et al. (2011) found that despite similar cognitive-task performance, patients with chronic pain associated with temporomandibular disorder had an increased task-evoked neural response in frontal temporal, parietal and insular regions compared with controls. Cognitive-task performance was also associated with an activation (rather than a typical deactivation) of the basal default mode network in pain patients, as well as reduced amygdala-cingulate connectivity compared with controls. Glass et al. (2011) also found no significant changes in a go/no-go task performance in FM patients compared with controls, but reported functional decrease in activation in brain regions including the motor cortices, the midcingulate and the inferior frontal gyrus, areas normally involved in attention and response inhibition. The authors also observed an increase in cortical activity which may act as a compensatory mechanism.

1.2.2 Brain morphology and cellular alterations in pain and cognition

Gross changes in brain morphology, and more subtle regional and cellular morphological changes, have been reported in various types of chronic pain. Patients suffering from chronic lower back pain and FM, for example, have significantly less total grey matter volume than age-matched healthy controls (Apkarian et al., 2004b; Kuchinad et al., 2007; Schweinhardt et al., 2008; Wood et al., 2009; Absinta et al., 2012; Ruscheweyh et al., 2011). A reduction in grey matter is a feature
of ageing, which is inherently associated with cognitive decline and in particular in the speed of information processing and executive functions (Salthouse, 1996; Salthouse et al., 1998). Loss of grey matter in chronic pain patients occurs at an increased rate compared with healthy controls (Kuchinad et al., 2007) and this may represent an acceleration of ageing-induced grey matter loss. Loss of grey matter may contribute to some of the cognitive deficits that have been described in chronic pain patients. Voxel-based morphometry has been used to examine region-specific changes in grey matter volume in chronic pain patients, with regions such as the IC, the ACC and the dorsolateral PFC consistently showing reduced volumes (Apkarian et al., 2004b; Kuchinad et al., 2007; Luerding et al., 2008; May, 2008; Schmidt-Wilcke et al., 2005, 2006, 2007; Valfre et al., 2008; Geha et al., 2008; Absinta et al., 2012). As discussed earlier in this section, the IC and the ACC are commonly implicated in both pain-experience processing and cognitive processing. The PFC is also critically involved in pain processing, comprising part of the pain neuromatrix, but in addition is involved in working memory and recognition, and is the neural centre of executive functioning (Kouneiher et al., 2009; Robbins, 1996; Gilbert and Burgess, 2008). Therefore, grey matter changes in the frontal or cingulate cortex could be associated with impaired working memory or executive functioning. Luerding and colleagues (2008) investigated this possibility by examining correlations between grey matter volumes and neuropsychological performance in chronic pain patients. They found that visual working memory was positively correlated with grey matter volume in the left dorsolateral PFC. More recently, alterations have also been observed in the morphology of the hippocampus in chronic pain patients. Zimmerman et al. (2009) found that hippocampal volumes were moderately decreased in chronic pain patients, while Mutso et al. (2012) showed a more robust decrease in hippocampal volume associated with chronic lower back pain and CRPS but not with osteoarthritis. Unfortunately, these techniques do not allow us to determine whether pain-related variance in grey matter results from changes in the number
of neurons, interneurons or glia, or from changes in cell size. Relatively few studies have investigated changes in white matter in chronic pain, though Buckalew et al. (2008, 2010) have shown decreases in white-matter volume (Buckalew et al., 2008) and in white-matter integrity (Buckalew et al., 2010) in the brains of older chronic pain patients. Alterations in the connectivity of white-matter tracts between important cognitive brain regions between have also been shown in CRPS (Geha et al., 2008). Animal models of neuropathic pain, including SNI, SNL and chronic constriction injury (CCI) models have been used to examine pain-related changes in brain morphology. A recent study by Seminowicz et al. (2009) used MRI to examine pain-related alterations in the brain of SNI rats and found that decreases in cortical volume in SI and SII regions, the ACC and the IC correlated with the degree of mechanical hyperalgesia. They also showed that SNI rats had decreased prefrontal cortex volume and that this decrease was temporally associated with onset of anxiety behaviours. These authors reported a decrease in the number of exits from closed arms as an index of anxiety behaviour, as opposed to more common measures such as the time spent on open arms or closed arms and the number of entries to open and closed arms. Thus, it is difficult to make direct comparisons with other studies measuring anxiety-like behaviour in rodents. As noted in the previous section, the prefrontal cortex is also vitally important in cognition, with particular relevance to executive function. Therefore, alterations in grey matter in this region may also have implications for cognitive functioning.

Pain-related changes at a cellular level have also been examined in rodent models of chronic pain. Cell-type specific increases in firing frequency of neurons in the ACC have been shown following peripheral nerve injury in mice (Cao et al., 2009). Length and branching of basal dendrites and spine density were increased in pyramidal neurons in acute slices of the medial PFC contralateral to nerve injury in the SNI model (Metz et al., 2009). It is possible that these anatomical changes in the PFC may contribute to cognitive impairment, in particular impaired flexibility such as that described by Leite-Almeida and colleagues (2009) following SNI (see
Section 1.1.2). Gonçalves et al. (2008) have also shown increases in amygdalar volume and increased neuronal proliferation in the amygdala of SNI rats.

The amygdala is highly involved in pain processing and is also a component of the limbic system. It is therefore thought to be important in the affective component of pain. Decreased neurotransmission in the amygdala, by administration of the gamma-aminobutyric acid (GABA)$_A$ receptor agonist muscimol or lesion of the central nucleus, has been shown to attenuate pain-induced avoidance behaviour, i.e., impaired affective processing of pain (Pedersen et al., 2007).

Although these latter studies are of relevance to memory acquisition, they are also associated with an affective component making it difficult to determine conclusively whether the pain-related deficits observed relate specifically to cognition per se or to altered affective state of the animal.

1.2.3 Synaptic plasticity in pain and cognitive impairment

One of the seminal theories linking pain and cognitive function is that neuroplastic changes occur in chronic pain and that resulting neural rewiring interferes with normal cognitive functioning. Long-term potentiation (LTP) is a type of synaptic plasticity in which synaptic function is enhanced following a high-frequency burst of presynaptic stimulation. Central sensitization and wind-up involve repeated high-frequency stimulation of nociceptors to increase spinal reflexes and excitability of central nociceptive circuits, a process similar to LTP. Central sensitization is thought to be a key step in the transition between acute and chronic pain. Pain-induced synaptic plasticity has also been shown to occur in higher brain regions with known roles in cognitive function. The hippocampus is traditionally associated with learning and memory functions. In particular, it is believed to facilitate memory consolidation via LTP. *Ex vivo* electrophysiology studies show that LTP is impaired in hippocampal slices from nerve-injured mice and rats (Kodama et al., 2007, 2011; Ren et al., 2011; Tanabe et al., 2008). Subregions of the hippocampal slices were stimulated and recording electrodes measured the field
excitatory post-synaptic potentials (fEPSPs) before and during high-frequency conditioning stimuli to induce LTP. The procedure is considered a basic cellular model for learning and memory, and the pain-related reduction in LTP may relate to the cognitive disturbances associated with chronic pain. Studies have also shown impaired LTP in slices taken from diabetic rats (Kamal et al., 1999; Tanabe et al., 2008). This result may mirror cognitive disturbances seen in the clinical diabetic population. However, it is important to note that STZ-induced hypersensitivity was confirmed in only one of these studies (Tanabe et al., 2008). Decreased short-term plasticity has also been demonstrated in hippocampal slices in the SNI model of neuropathic pain. Plasticity, induced by high-frequency pain stimuli, was enhanced in the amygdala (Bird et al., 2005; Fu et al., 2008; Neugebauer et al., 2003), the ACC (Zhuo, 2006, 2007) and the hippocampus (Zhao et al., 2009) in rodent models of prolonged inflammatory pain. Pain was also associated with increased synaptic efficacy in the hippocampus and the amygdala (Ji et al., 2010; Zhao et al., 2009). Ikeda et al. (2007) demonstrated an increase in the amplitude of evoked post-synaptic potentials in central-amygdala neurons in SNL rats, compared with sham controls. An elegant electrophysiological study by Ji et al. (2010) demonstrated that interaction between the amygdala and the PFC contributes to pain-related cognitive impairment in a rodent gambling task. Arthritic pain-related behaviour was associated with impaired performance on the task, as well as increased neuronal excitability and synaptic transmission in the basolateral nucleus of the amygdala. Pharmacological deactivation of the basolateral amygdala restored normal task performance and increased activity in neurons of the medial PFC. Thus, the authors propose that an “amygdala-driven” deactivation of the PFC is responsible for pain-related cognitive impairment in this task. These results provide evidence for pain-induced alterations in plasticity and suggest that there may be a cellular basis for the cognitive impairments associated with chronic pain. The study by Ji et al. (2010) is particularly important as it is the first to propose a mechanism that directly relates pain to cognitive...
imperative by combining behavioural, electrophysiological and pharmacological approaches. More recent studies also lend support to a theory of pain-related deactivation of the PFC and have suggested a role for the glutamatergic system (Ji and Neugebauer, 2010; Sun and Neugebauer, 2011). It is unclear whether this supraspinal pain-induced plasticity is an extension of the central sensitization that occurs in the spinal cord. As altered synaptic plasticity may play a role in pain-related cognitive function, the study of synaptic proteins such as synaptophysin may also be interesting in the case of pain and cognition and their interaction. Synaptophysin is the most abundant synaptic vesicle membrane protein, comprising approximately 7% of the total vesicle protein. Encoded by the SYP gene, it has a molecular weight of 33.8 kDa and contains four transmembrane domains (Figure 1.3) (Douglass et al., 2008). The ubiquitous expression of synaptophysin suggests that it may have an important role in synaptic vesicle exocytosis (Kwon and Chapman, 2011). However, synaptophysin knockout mice appeared to show normal synaptic transmission (McMahon et al., 1996). This finding may be due to compensation by other synaptic proteins and double knockout of synaptophysin and synaptogyrin was shown to impair hippocampal CA1 plasticity (Janz et al., 1999). Synaptogyrin and synaptophysin are similar in their structure and activation, suggesting a common evolutionary origin, though their precise mechanism in synaptic plasticity is poorly understood. More recent research has shown that synaptophysin may be involved in vesicle endocytosis and trafficking (Kwon and Chapman, 2011). The level of synaptophysin is thought to correlate with the number of synaptic connections (Calhoun et al., 1996), and is altered in models of cognitive impairment, both age-related (Benice et al., 2006; Calhoun et al., 1998) and disease-related (Seabrook et al., 1999; King and Arendash, 2002). Synaptophysin knockout mice also showed deficient novel object recognition and spatial memory (Schmitt et al., 2009). Synaptophysin is altered in the periphery and spinal cord in animal models of chronic pain (Peng et al., 2010; Chou et al., 2002; Sun et al., 2006; Jaken et al., 2010; Lin et al., 2011). Recently a pain-related de-
crease in synaptophysin-positive boutons was observed in the CA1 region of the hippocampus in a neuropathic pain model associated with memory impairments (Ren et al., 2011).

![Transmembrane behaviour of synaptophysin](image)

Figure 1.3: Transmembrane behaviour of synaptophysin (Douglass et al., 2008); each circle represents an amino acid.

### 1.2.4 Neurotransmitters and receptors in pain and cognition

#### 1.2.4.1 Glutamate

Several neurotransmitter systems are commonly involved in both pain processing and cognition. Glutamate transmission through the N-methyl-D-aspartic acid (NMDA) receptor is essential for learning and memory through LTP (Brown et al., 1988; Rison and Stanton, 1995). NMDA receptors are also implicated in central sensitization and wind-up (Scholz and Woolf, 2002). As described above, pain-related plasticity in higher brain regions is a feature of chronic pain models, and while it is unclear whether this is an extension of the central sensitisation occurring at the level of the spinal cord, the critical involvement of NMDA (Bird et al., 2005; Fu et al., 2008; Zhuo, 2006, 2007), AMPA (Zhao et al., 2009) and metabotropic (Ji
et al., 2010; Neugebauer et al., 2003) glutamate receptors suggests some mechanistic similarity. One possibility is that pain-induced synaptic plasticity co-occurring with learning/memory-related LTP could result in an overall interference effect which might account for cognitive impairment at a molecular level. Analgesic drugs targeting the glutamatergic system are limited by their cognitive effects. NMDA receptor antagonists such as ketamine show analgesic activity, but also cause CNS effects including visual and auditory disturbances and hallucinations, feelings of unreality and feelings of detachment from the body (Chizh and Headley, 2005). Blockade of metabotropic glutamate receptors (mGluR1 subtype with the selective antagonist A-841720) has been shown to reduce inflammatory pain and allodynia in animal models but impair function in the Y-maze and water maze tests of cognition (El-Kouhen et al., 2006).

1.2.4.2 GABA

GABA is an inhibitory neurotransmitter and can dampen neuronal activity by inhibiting the release of other neurotransmitters (Enna and McCarson, 2006). GABA can therefore slow sensory transmission and reduce perceived pain. In particular, loss of the inhibitory function of GABA in the spinal cord may contribute to pathological pain (Enna and McCarson, 2006). As GABA is a ubiquitous transmitter, it can also slow cognitive processes and cause sedation, linking pain and cognitive systems. Hyperactivation of the basolateral amygdala in an inflammatory pain model increased GABAergic tone in the prefrontal cortex, and was associated with pain-induced impairment on an emotional decision-making task in rats (Ji et al., 2010). GABA receptor agonists have analgesic efficacy in acute and neuropathic pain models (Malan et al., 2002), but sedative effects and impaired locomotor activity have limited their use as therapeutic agents. Targeting specific subtypes of GABA\textsubscript{A} receptors (containing \(\alpha 2\) and/or \(\alpha 3\) subunits) reduced inflammatory and nociceptive pain without causing adverse sedative effects (Knabl et al., 2008; Möhler, 2009), and selective inverse agonists of the \(\alpha 5\)
subunit enhance spatial learning in animal behavioural tests of cognitive function (Maubach, 2003; Möhler, 2009). Co-expression and activation of these receptor subtypes may be involved in the interaction between pain and cognition, though further research is warranted.

1.2.4.3 Acetylcholine

Acetylcholine has classically been recognised as one of the most important mediators of cognitive processes such as learning and memory (Drachman and Leavitt, 1974; Lucas-Meunier et al., 2003; McGuire, 1990). There is less evidence supporting a role for cholinergic transmission in pain. However, this transmitter is thought to play a role, both directly and indirectly, in the descending inhibitory control of pain (Millan, 2002), and neuronal nicotinic and muscarinic acetylcholine receptors have been implicated in pain transmission. Studies have shown that nicotinic receptor agonists, such as epibatidine and nicotine itself, are analgesic when administered centrally (Jones and Dunlop, 2007). Muscarinic receptor agonists such as vendacycline, oxotremorine, CMI-936 and CMI-1145, have also shown potent analgesic action (Tata, 2008; Wess et al., 2007). Furthermore, neostigmine, which inhibits the degradation of acetylcholine, reversed allodynia and hyperalgesia in rat models of neuropathic pain (Jones and Dunlop, 2007). As is the case for GABA, specific cholinergic receptor subtypes may be important in the interaction between pain and cognition. A recently characterised partial agonist of the nicotinic acetylcholine receptor α7 subtype, JN403, was found to have antinociceptive effects in rats and improved social learning in a social recognition paradigm (Feuerbach et al., 2008). Thus, the cholinergic system may subserve pain/cognition interactions.

1.2.4.4 Endocannabinoids

The endocannabinoid system is involved both in pain (Graham et al., 2009; Hohmann and Suplita, 2006) and in the cognitive processes of learning and mem-
ory (Riedel and Davies, 2005; Solowij and Battisti, 2008). Inflammatory and neuropathic pain were found to be associated with increased levels of endocannabinoids in the rat PAG (Petrosino et al., 2007; Walker et al., 1999), and PAG cannabinoid CB1 receptor expression was increased in a rat model of diabetic hyperalgesia (Mohammadi-Farani et al., 2010). In the ACC, endocannabinoid levels and cannabinoid CB1 receptor expression were unchanged in the mouse CCI model of neuropathic pain, but receptor coupling/functionality (measured by $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding) was decreased (Hoot et al., 2010). Therefore, pain is associated with region-specific alterations in endocannabinoid signalling in brain regions associated with cognitive processing. Cannabinoid receptor agonists are associated with impaired performance in spatial learning and recognition memory tasks (Ferrari et al., 1999; Kosiorek et al., 2003; Suenaga and Ichitani, 2008; Suenaga et al., 2008; Takahashi et al., 2005), while cannabinoid receptor antagonists improve performance in cognitive tests (Lichtman, 2000; Wise et al., 2008; de Bruin et al., 2010). Cannabinoid ligands have also demonstrated antinociceptive effects in acute, inflammatory and neuropathic pain models (Finn and Chapman, 2004; Manzanares et al., 2006), and have a synergistic analgesic effect when co-administered with opioids (Welch and Eads, 1999). However, there is a paucity of studies directly investigating potential involvement of the endocannabinoid system in pain-related cognitive impairment.

1.2.4.5 Aspartate

N-acetyl aspartate is a precursor of the excitatory neurotransmitter aspartate and is known to be localized to neurons involved in synaptic processes. Reduced levels of this molecule were found in the PFC of patients with chronic lower back pain (Grachev et al., 2000). Attentional interference in the Stroop task is associated with reduced N-acetyl aspartate in the ACC (Grachev et al., 2001). Decreased N-acetyl aspartate occurs in a number of degenerative disorders, and is thought to be indicative of neuronal loss. These changes in cortical organisation may
again contribute to impairments in cognitive functioning. Grachev et al. (2000) also found reductions in glucose in the PFC of chronic pain patients, which may suggest a decrease in metabolic rate in this region, which in turn might be expected to affect performance on cognitive tests.

1.2.4.6 Opioids

Opioids remain among the most commonly used and most effective classes of analgesic medications. Drugs such as morphine act on the opioidergic system by activating $\mu$, $\delta$ and/or $\kappa$ opioid receptors and by initiating various downstream signal cascades (Zöllner and Stein, 2007). Endogenous opioids are thought to play a key role in the descending inhibitory pain pathway, capable of inducing potent analgesia (Millan, 2002). In addition to their pain-relieving effects, opioids are associated with adverse effects such as sedation and mental clouding. These cognitive-associated effects are thought to relate to the presence of opioid receptors in brain regions involved in learning, memory and attention such as the ACC (Jamison et al., 2003; Jones et al., 1991; Petrovic et al., 2010). However, the relationship between opioids and cognitive function appears to be complex, and there is evidence to suggest differential effects of opioids in the presence or absence of chronic pain. While cognitive impairment was observed following opioid administration in healthy volunteers and in naïve animals, morphine and other opioid analgesics were actually found to improve cognitive impairment in chronic pain patients and in animal models of chronic pain (see Section 1.2.7, Table 1.4). Changes in opioid receptor binding have been demonstrated using imaging studies in various clinical chronic pain conditions in brain regions that modulate pain, including the dorsolateral PFC, the ACC, the PAG and the thalamus (Jones et al., 1994, 1999, 2004; Willoch et al., 2004), and opioid receptor-mediated G-protein activity in the thalamus was reduced in a mouse model of chronic neuropathic pain (Hoot et al., 2011). It is unclear whether these changes relate to a decrease in receptor number, altered binding properties or an increase in the level of endogenous opioid
peptides. The role of the opioidergic system in the interaction between pain and cognition has been studied extensively in the context of top-down attentional or cognitive pain control. Placebo analgesia is mediated by endogenous activity at μ opioid receptors, and opioids are also thought to be involved in endogenous analgesia associated with distraction, anticipation and hypnosis (Bingel and Tracey, 2008; Petrovic et al., 2002; Zubieta et al., 2005). The opioid system, therefore, represents an interesting convergence between pain and cognitive processing systems. Its involvement in pain-related impairment of cognitive function has not been studied extensively to date, and this warrants further investigation.

1.2.4.7 Monoamines

The monoaminergic system plays a key role in expression of pain (Kayser et al., 2007; Mansikka et al., 2005; Millan, 2002; Papaleo et al., 2008; Plaźnik et al., 1985), and pain has been shown to be associated with alterations in monoamine signalling in cognitive-associated brain regions (Finn et al., 2006; Omote et al., 1998; Wood, 2008; Wood et al., 2009; Wood and Holman, 2009; Wood et al., 2007a,b; Jarcho et al., 2012). In addition, tricyclic antidepressants (TCAs) which block reuptake of noradrenaline and serotonin are efficacious in the treatment of chronic pain (McCleane, 2003). There is also an established role for monoamines in cognitive abilities such as attention and impulse control, both in humans and in rodents (Bunsey and Strupp, 1995; Scholes et al., 2007; Bari et al., 2009; Robinson, 2012). Due to their shared role in cognitive function and pain processing, therefore, monoamines may be implicated in the mechanisms underlying pain-related cognitive impairment. However, few studies have investigated monoamine neurotransmitters in both pain and cognition. An early study by Plaźnik et al. (1985) found that intra-amygdalar serotonin reduced pain behaviour but impaired retention of a passive avoidance response. Mutations in the gene encoding catechol-O-methyltransferase (COMT), an enzyme involved in metabolism of monoamines, resulted in decreased pain sensitivity in transgenic mice, but also impaired work-
ing and recognition memory (Papaleo et al., 2008). Pain-related impairment of
cognitive performance on a rodent gambling task was found to be associated with
a decrease in levels of dopamine and its metabolites in the orbitofrontal cortex
(Pais-Vieira et al., 2009b). A reduction in dopamine and serotonin metabolites in
the prefrontal cortex of rats following expression of distraction-induced analgesia
has also been reported (Ford et al., 2008). Dopamine is thought to be impor-
tant in neuronal integrity (Bozzi and Borrelli, 2002; Bozzi et al., 2000), and so
dysfunctional dopamine signalling may contribute to neuronal loss, which may in
turn affect cognition (Nakajima et al., 2012).

The neurotransmitter systems discussed above are potentially altered in chronic
pain by increased or decreased activation, or by disinhibition. As these systems
are also crucial to normal cognitive functioning, alterations caused by chronic
pain may negatively affect cognition. Further research is needed to confirm this
hypothesis, and to fully investigate the relative contribution of each system and
the interaction between them.

1.2.5 Glial cells and cytokines in pain and cognition

Chronic pain is also associated with changes in neural mediators other than clas-
sical neurotransmitters. These include cytokines, glial cells, enzymes and neu-
rotrophic factors. Dysregulation of these mediators could also affect cognitive
processing ability.

Recent research has highlighted the importance of glial cells in relation to pain
(Inoue and Tsuda, 2009; McMahon et al., 2005; Miller et al., 2009), and in pro-
cesses such as synaptic plasticity, important for learning and memory (Bains and
Oliet, 2007). Furthermore, activated glial cells can release a variety of cytokines
and neurotrophic factors which also modulate neural processes involved in pain
and cognition (Covey et al., 2000; Ren and Dubner, 2008; Tanaka et al., 2006).
The SNL model of neuropathic pain in mice is associated with increased activa-
tion of astroglia in the ACC (Kuzumaki et al., 2007), a region which, as discussed
previously, plays a vital role in pain and cognition. In addition, administration of the glial modulating drug, propentofylline, directly into the cingulate cortex 24 hours before ligation had antiallodynic and antihyperalgesic effects (Kuzumaki et al., 2007). A role for glial cells in chronic pain and associated mental fatigue has been proposed by Hansson and Rönnbäck (2004). These authors suggest that pro-inflammatory cytokines, released by activated microglia, cause impaired uptake of the neurotransmitter glutamate by astroglia. This leads to an accumulation of glutamate in the extracellular space which in turn diminishes synaptic efficiency, reduces the precision of glutamate signalling, and eventually reduces stimulus-induced glutamate release. One may speculate that these changes might underlie poor cognitive performance in chronic pain, though further research is necessary to confirm this theory. Cytokines are signalling molecules expressed in most pain states, but are most classically associated with inflammation. Cytokines are now also thought to be involved in synaptic plasticity and memory consolidation (Depino et al., 2004). Levels of pro-inflammatory cytokines, IL-1β and IL-6, are increased in the cerebrospinal fluid (CSF) of chronic pain patients (Alexander et al., 2005). In the rat SNI model of chronic neuropathic pain, levels of the pro-inflammatory cytokine, IL-1β, were elevated in the brainstem and in the right prefrontal cortex (PFC) at 10 days post injury (Apkarian et al., 2006). Expression of IL-1β was also increased in the hippocampus of nerve-injured rats (del Rey et al., 2011), while TNF-α was increased in the hippocampal tissue, the CSF, and the plasma (Ren et al., 2011). Direct intrahippocampal or intracerebroventricular injection of TNF-α in rats was also found to mimic pain-related deficits in memory in the eight-arm radial maze (Ren et al., 2011), while genetic deletion of TNF-α in mice attenuated the pain-related deficit (Ren et al., 2011). Pain-induced changes in cytokine expression in brain regions such as the PFC may, therefore, affect cognitive functioning by altering synaptic processes.
1.2.6 Enzymes in pain and cognition

The enzyme calcium (Ca$^{2+}$)/calmodulin-dependent kinase II (CAMKII) is an interesting molecule in the context of pain/cognition interaction. Overexpression of forebrain (ACC, hippocampus and amygdala) CAMKII in transgenic mice is associated with antinociceptive effects and decreased long-term depression (LTD) in the ACC (Wei et al., 2006). LTD, or weakening of synaptic efficiency, is induced by a low-frequency prolonged stimulation and is considered to be the reverse of LTP. It is thought to be involved in erasing old memories and allowing the formation of new memories. Therefore, impaired LTD in the anterior cingulate cortex (ACC) may inhibit the removal of pain-related memories. However, it is important to note that since LTD weakens synaptic efficiency, inhibition of this process may in fact facilitate cognition. In a separate experiment, mutations in the gene encoding CAMKII inhibited LTP in the hippocampus and impaired spatial learning in the water maze (Giese et al., 1998). Thus, supraspinal CAMKII appears to be important in both pain and cognition, though its potential role in the interaction between pain and cognition is yet to be investigated directly.

Neuropathic pain was associated with increased expression of caspases in the prefrontal cortex (Neugebauer et al., 2009). Caspases are a family of cysteine protease enzymes and are thought to be involved in regulating the release of cytokines, as well as playing a central role in apoptosis (Thornberry and Lazebnik, 1998). Expression of the effector caspase, caspase 3, may reflect cell death associated with neuropathic pain in the prefrontal cortex, which could contribute to cognitive dysfunction (Neugebauer et al., 2009).

A recent study has shown that expression and phosphorylation of the extracellular signal-related kinases (ERK) 1 and 2 were altered in the hippocampus in a model of neuropathic pain in mice. Significantly, the model was also associated with pain-related dysfunction of the hippocampus (Mutso et al., 2012). Thus further investigation of these enzymes in relation to pain and cognitive impairment is also warranted.
1.2.7 Neurotrophic factors in pain and cognition

Brain-derived neurotrophic factor (BDNF) plays a critical role in synaptic plasticity, memory processes and storage of long-term memory (Bekinschtein et al., 2008a,b; Yamada and Nabeshima, 2003). BDNF has also been found to enhance neurogenesis (Binder and Scharfman, 2004), thought to be important for hippocampal-dependent learning and memory. Impaired neurogenesis has also been demonstrated in models of chronic pain (Mutso et al., 2012; Terada et al., 2008). BDNF expression in the hippocampus is decreased in rat models of both neuropathic pain and inflammatory pain (Duric and McCarson, 2006; Hu et al., 2010). Moreover, pharmacological alleviation of neuropathic pain-related behaviour was associated with increased levels of BDNF and reversal of pain-related cognitive impairment. These findings suggest that pain-induced decreases in BDNF may mediate cognitive impairment.

The literature reviewed provides support for a model of pain-related cognitive impairment based on three main theories: (1) limited resources, (2) altered neuroplasticity and (3) dysregulated neurochemistry. Pain utilises cognitive resources, alters neural plasticity and affects expression and activity of a variety of chemical and cellular neuromediators. These effects, which are not necessarily mutually exclusive, occur across a complex network of interconnected cognition-related brain regions to produce a net cognitive impairment. This theoretical model is illustrated in Figure 1.4. Though the model presented is necessarily speculative at this time, it provides a framework for the design of studies aimed at elucidating the neural mechanisms involved in pain-related cognitive impairment.
to enhance neurogenesis (Binder and Scharfman, 2004), thought to be important for hippocampal-dependent learning and memory. BDNF expression in the hippocampus was decreased in models of both neuropathic pain and inflammatory pain (Duric and McCarson, 2006; Hu et al., 2010). Moreover, pharmacological alleviation of neuropathic pain-related behaviour was associated with increased levels of BDNF and reversal of pain-related cognitive impairment. These findings suggest that pain-induced decreases in BDNF may mediate cognitive impairment. The literature reviewed above provides support for a model of pain-related cognitive impairment based upon three main theories; (1) limited resources, (2) altered neuroplasticity and (3) dysregulated neurochemistry. Pain utilises cognitive resources, alters neural plasticity and affects expression and activity of a variety of chemical and cellular neuromediators. These effects, which are not necessarily mutually exclusive, occur across a complex network of interconnected cognition-related brain regions to produce a net cognitive impairment. This theoretical model is illustrated in Fig. 1. Though the model presented is necessarily speculative at this time, it may provide a framework for the design of further studies aimed at elucidating the neural mechanisms involved in pain-related cognitive impairment.

6. The effects of analgesic treatments for chronic pain on cognitive function

Treatment of chronic pain represents a major challenge for healthcare professionals. Multimodal analgesia is often necessary to achieve adequate pain relief and protracted medical management is frequently required in patients suffering from chronic pain. Current strategies for the management of pain focus mainly on its sensory component. Pharmacological interventions treat inflammation and associated sensitization of nociceptors (non-steroidal anti-inflammatory drugs; NSAIDs), enhance endogenous analgesic mechanisms (opioids, TCAs) or dampen the excitability of pain-transmitting neurons (opioids, anticonvulsants). Some of these agents may be associated with cognitive dysfunction de novo, or further exacerbation of existing cognitive impairment. As illustrated in Table 3, the cognitive profiles associated with analgesic drugs, are complex and varied. Opioid, TCA and anticonvulsant drugs have all been related to impaired cognitive function in various domains (see Table 3), though inconsistently. Other studies have found no adverse cognitive effects with these medications or have actually found improvements in cognitive function, particularly in chronic pain patients and in animal models of chronic pain (Boyette-Davis et al., 2008; Hu et al., 2010; Jamison et al., 2003; Millecamps et al., 2004; Tassain et al., 2003). Proposed explanations for these findings include development of tolerance to the adverse cognitive effects, as well as the idea that effective pain relief may reverse pain-induced cognitive impairment. Combined D9-tetrahydrocannabinol (THC) and cannabidiol was not shown to affect cognition and NSAIDs were found to improve cognition (Table 3).

In assessing cognitive function in chronic pain patients, the majority of studies fail to adequately control for confounds related...
1.3 The effects of analgesic treatments for chronic pain on cognitive function

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As illustrated in Table 1.4, the cognitive profiles associated with analgesic drugs, are complex and varied. Opioid, TCA and anticonvulsant drugs have all been related to impaired cognitive function in various domains (see Table 1.4), though inconsistently. Other studies have found no adverse cognitive effects with these medications or have actually found improvements in cognitive function, particularly in chronic pain patients and in animal models of chronic pain (Boyette-Davis et al., 2008; Hu et al., 2010; Jamison et al., 2003; Millecamps et al., 2004; Tassain et al., 2003). Proposed explanations for these findings include development of tolerance to the adverse cognitive effects, as well as the idea that effective pain relief reverses pain-induced cognitive impairment. Combined $\Delta^9$-tetrahydrocannabinol (THC) and cannabidiol was not shown to affect cognition in multiple-sclerosis patients, while $\Delta^9$-THC alone impaired spatial learning and memory in rodents. Other experimental cannabinoid drugs have also been shown to affect cognition (see Section 1.2.4.4). Improved cognition has been demonstrated following treatment with NSAIDs (Table 1.4).

In assessing cognitive function in chronic pain patients, the majority of studies fail to adequately control for confounds related to analgesic medication, which
represents a serious limitation in determining the value of these studies. Of the clinical studies outlined in Table 1.1, only one study controlled fully for the effects of medication by including a non-medicated comparison group \((n = 21)\) as well as drug-treated groups (Sjøgren et al., 2005). These authors found that patients taking oral opioids performed more poorly than the non-medicated group on the PASAT (which measures attention, concentration, working memory and speed of information processing). Patients in another study were free of any medication (Povedano et al., 2007), and some studies were performed following a drug washout period (Antepohl et al., 2003; Park et al., 2001). In some cases, authors excluded patients taking opioid or antidepressant drugs (Harman and Ruyak, 2005), or corrected statistically for the effects of medication status (Eccleston, 1995; Karp et al., 2006; Lee et al., 2010; Oosterman et al., 2011). The inclusion of non-medicated control groups may not always be possible in studies involving chronic pain patients for ethical reasons. However, many analgesic drugs are known to affect cognition and this property could alter or confound the results of neuropsychological assessment in chronic pain patients.

Cognitive impairment associated with analgesic medication may, therefore, further exacerbate pain-related cognitive impairment, but effective analgesia may alleviate pain and its associated cognitive impairment. Examining the effects of analgesics on cognition and pain-related cognitive impairment may also help to elucidate the mechanisms underlying cognitive impairment in chronic pain. Analgesic reversal of pain-related cognitive impairment, as occurs with opioids and amitriptyline (Boyette-Davis et al., 2008; Hu et al., 2010; Jamison et al., 2003; Millecamps et al., 2004; Tassain et al., 2003), is an important factor in defining a causal relationship between chronic pain and cognitive impairment.
Table 1.4: Summary of known cognitive effects of approved analgesic medication.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Effect on cognitive function</th>
<th>Type of cognitive task</th>
<th>Type of subject</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opioids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine</td>
<td>↓</td>
<td>Verbal processing</td>
<td>Healthy human volunteers</td>
<td>Kerr et al. (1991)</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>↓</td>
<td>Attention, verbal learning, working memory, reaction time</td>
<td>Healthy human volunteers</td>
<td>Cherrier et al. (2009)</td>
</tr>
<tr>
<td>Misc. opioids</td>
<td>↓</td>
<td>Attention</td>
<td>Chronic pain patients</td>
<td>Sjøgren et al. (2005)</td>
</tr>
<tr>
<td>Misc. opioids</td>
<td>↓</td>
<td>Reaction time</td>
<td>Cancer pain patients</td>
<td>Banning et al. (1992)</td>
</tr>
<tr>
<td>Misc. Opioids</td>
<td>↔</td>
<td>Concentration, verbal learning, psychomotor function and speed</td>
<td>Post-herpetic neuralgia patients</td>
<td>Raja et al. (2002)</td>
</tr>
<tr>
<td>Morphine</td>
<td>↑</td>
<td>Attention and psychomotor speed</td>
<td>Chronic pain patients</td>
<td>Tassain et al. (2003)</td>
</tr>
<tr>
<td>Oxycodone, fentanyl</td>
<td>↑</td>
<td>Visuomotor speed and coordination</td>
<td>Lower back pain patients</td>
<td>Jamison et al. (2003)</td>
</tr>
<tr>
<td>Morphine</td>
<td>↓</td>
<td>Operant learning and spatial memory</td>
<td>Rat</td>
<td>Wang et al. (2006)</td>
</tr>
<tr>
<td>Morphine</td>
<td>↓</td>
<td>Spatial memory</td>
<td>Rat</td>
<td>Ma et al. (2007)</td>
</tr>
<tr>
<td>Morphine</td>
<td>↑</td>
<td>Operant non-matching to position task</td>
<td>CFA-treated rats</td>
<td>Cain et al. (1997)</td>
</tr>
<tr>
<td>Morphine</td>
<td>↑</td>
<td>Operant non-matching to position task</td>
<td>CFA-treated rats</td>
<td>Lindner et al. (1999)</td>
</tr>
<tr>
<td>Morphine</td>
<td>↑</td>
<td>Operant attention task</td>
<td>Formalin-treated rats</td>
<td>Boyette-Davis et al. (2008)</td>
</tr>
<tr>
<td>Morphine</td>
<td>↑</td>
<td>Object recognition</td>
<td>Rat model of visceral pain</td>
<td>Millecamps et al. (2004)</td>
</tr>
<tr>
<td>TCAs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>↓</td>
<td>Memory encoding and retrieval</td>
<td>Depressed patients</td>
<td>Spring et al. (1992)</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>↓</td>
<td>General intelligence and psychomotor performance</td>
<td>Post-herpetic neuralgia patients</td>
<td>Raja et al. (2002)</td>
</tr>
<tr>
<td>Misc. TCAs</td>
<td>↔</td>
<td>General cognition (MMSE)</td>
<td>Depressed patients</td>
<td>Podewils and Lyketsos (2002)</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>↓</td>
<td>Recognition memory</td>
<td>Rat</td>
<td>Burgos et al. (2005)</td>
</tr>
</tbody>
</table>

Continued on next page...
### Table 1.4 (continued from previous page)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Effect on cognitive function</th>
<th>Type of cognitive task</th>
<th>Type of subject</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>↓</td>
<td>Visuo-spatial memory</td>
<td>Rat</td>
<td>Naudon et al. (2007)</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>↑</td>
<td>Spatial learning and memory</td>
<td>Rat model of neuropathic pain</td>
<td>Hu et al. (2010)</td>
</tr>
<tr>
<td><strong>Anticonvulsants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↑</td>
<td>Memory retention</td>
<td>Mouse</td>
<td>Acosta et al. (2000)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↔</td>
<td>Memory retention</td>
<td>Rat</td>
<td>de-Paris et al. (2000)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↔</td>
<td>Spatial memory</td>
<td>Rat</td>
<td>Shannon and Love (2004)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↔</td>
<td>Attention</td>
<td>Rat</td>
<td>Shannon and Love (2005)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↓</td>
<td>Spatial learning</td>
<td>Rat model of diabetes</td>
<td>Lindner et al. (2006)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↑</td>
<td>Concentration, memory and reaction times</td>
<td>Healthy human volunteers</td>
<td>Saletu et al. (1986)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↓</td>
<td>Memory</td>
<td>Healthy human volunteers</td>
<td>Salinsky et al. (2010)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↔</td>
<td>Attention, psychomotor speed, language and memory</td>
<td>Healthy human volunteers</td>
<td>Martin et al. (1999)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↓</td>
<td>Psychomotor function</td>
<td>Older healthy human volunteers</td>
<td>Martin et al. (2001)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↔</td>
<td>Memory and psychomotor function</td>
<td>Epilepsy patients</td>
<td>Leach et al. (1997)</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>↑</td>
<td>Attention</td>
<td>Epilepsy patients</td>
<td>Mortimore et al. (1998)</td>
</tr>
<tr>
<td>Pregabalin</td>
<td>↓</td>
<td>Attention and arousal</td>
<td>Healthy volunteers</td>
<td>Hindmarch et al. (2005)</td>
</tr>
<tr>
<td>Pregabalin</td>
<td>↓</td>
<td>Episodic memory</td>
<td>Epilepsy patients</td>
<td>Ciesielski et al. (2006)</td>
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<td><strong>Cannabinoids</strong></td>
<td></td>
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<tr>
<td>Δ⁹-THC/cannabidiol</td>
<td>↔</td>
<td>Concentration and information processing</td>
<td>Multiple sclerosis patients</td>
<td>Wade et al. (2004)</td>
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<td>Δ⁹-THC</td>
<td>↓</td>
<td>Spatial learning and memory</td>
<td>Mice</td>
<td>Tselnicker et al. (2007)</td>
</tr>
<tr>
<td><strong>NSAIDs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>↑</td>
<td>Global cognitive performance</td>
<td>Elderly pain patients</td>
<td>Grodstein et al. (2008)</td>
</tr>
</tbody>
</table>

Table 1.4: Summary of known cognitive effects of approved analgesic medication. Control subjects were untreated, placebo-treated or vehicle-treated (in the case of animal studies).
1.4 The influence of ageing on pain and cognition

The effect of ageing on the interaction between pain and cognition is an important field of research due to the ageing population, and the expectation that the demand for pain management services will increase in the near future. The overall prevalence of chronic pain in older adults is higher than in the general population, though this relationship is complex and may differ across various chronic pain subtypes (Gagliese, 2009). Older age has consistently been associated with a decline in cognitive function (Salthouse, 2009, 2011). The relationship between age and pain sensitivity is more complex, with some studies providing evidence for age-related increases or decreases while other studies report no age-related changes (Gagliese, 2009). Discrepancies in the findings commonly relate to specific dimensions of pain. Some authors found age-related effects on sensory descriptors (“psychometric properties”) of pain scales but no differences in pain intensity across age (Gagliese and Melzack, 2003), while others reported decreased pain severity and interference in older adults but no age-related effects on aspects of depression, disability, life control and general activity (Turk et al., 1995). These variations may be influenced by differences in the measurement scales. Age-related differences in response to experimental nociceptive stimuli have also been demonstrated. Temporal summation of electrical stimuli appeared to occur at a lower frequency in older participants compared with the younger group, resulting in increased pain ratings (Farrell and Gibson, 2007). Furthermore, secondary hyperalgesia following application of topical capsaicin was more persistent in an older group of subjects compared with young subjects, despite similar primary hyperalgesia and levels of peak sensitivity in the secondary region (Zheng et al., 2000). Differences in pain sensitivity in rodents have also been shown primarily in supraspinally-mediated tests of nociception (for review, see Gagliese and Melzack 2000). An increase with age was observed in the stimulus intensity or duration to elicit a response in the hot-plate test and pressure- or shock-induced vocalisation tests. In addition, researchers have found that the relationship between age and increased pain sen-
sitivity in rodents is not straightforward: for example, in certain persistent pain models (formalin) a peak in pain sensitivity was observed in “mid-aged” rats (18 months) compared with both younger and older cohorts (Gagliese and Melzack, 1999). In models of neuropathic pain, nerve transection and constriction were associated with greater mechanical allodynia in mid-aged rats (16–18 months), while in nerve ligation, mid-aged rats (15 months) actually showed less mechanical and thermal hyperalgesia (Gagliese and Melzack, 2000). It is important to note that there is no widely accepted classification of young, mid-age and old age in the rat, and the precise age of the rats within classifications may differ across studies. The mechanisms by which age impacts on pain sensitivity are thought to include age-related changes in neurochemical and neuroendocrine mediators, changes in synaptic plasticity (Leite-Almeida et al., 2009), degeneration of descending inhibitory mechanisms (Iwata et al., 1995), and increase in the number, size and responsivity of spinal nociceptors (Gagliese and Melzack, 2000; Kitagawa et al., 2005). As discussed in Section 1.2.2 above, alterations in brain morphology have been observed in models of chronic pain, both clinically and preclinically, and these have been likened to an acceleration of the grey matter decline that occurs naturally with ageing. This may also provide an insight into the mechanism by which age affects pain sensitivity. The inconsistencies within studies investigating the effect of age on pain sensitivity are most likely due to model selection and methodological differences. However, age does appear to have a modulatory effect on pain sensitivity and is therefore an important consideration when considering the effects of pain on cognition.

A number of research studies have investigated pain-related cognitive impairment specifically in older participant groups (Karp et al., 2006; Buckalew et al., 2008; Scherder et al., 2008; Oosterman et al., 2009; Buckalew et al., 2010), or have accounted experimentally or statistically for age (see Table 1.1). Few authors have directly investigated the effect of ageing on the pain/cognition interaction, though Oosterman et al. (2011) found that increased age did not have an additive effect
on pain-related memory impairment. A study by Turk et al. (1995), however, did show that age significantly affected the relationship between pain and depression, and it is possible that the relationship between pain and cognition may be similarly affected. A study by Leite-Almeida et al. (2009) investigating cognitive performance in a model of chronic neuropathic pain in rats also demonstrated the importance of age. These authors found impairments in cognitive flexibility in rodents that had undergone SNI, but the deficit was specifically observed in mid-aged (9 months old) rats, and not in young or old rats (Figure 1.5). Overall, the influence of ageing on pain and cognition appears to be complex but significant and has therefore been taken into account in subsequent chapters of this thesis.

Figure 1.5: Effect of spared-nerve injury (SNI) on spatial reversal in the Morris water maze. Mid-aged SNI rats spent significantly more time in the old platform location (east quadrant of maze) than did age-matched sham controls, and conversely spent less time in the new platform location (west quadrant). This effect was not observed in other age cohorts. E: east; W: west. Figure kindly provided by H. Leite-Almeida (personal communication, May 29, 2012).
1.5 Overall objectives and experimental approach

The primary objective of the work presented herein was to improve our understanding of the phenomenon of pain-related cognitive impairment. The goal of the preclinical experiments was to establish a working rodent model (demonstrating face validity) of pain-related cognitive impairment, and to use this model to probe a subset of potential mechanisms involved. The clinical investigation was designed to build on and extend the existing literature by comparing a subset of cognitive functions not hitherto investigated in patients with neuropathic pain or radiculopathy versus controls. This work also attempted to control for common confounding variables (see Section 1.1.3) and to allow for comparison with preclinical findings in order to draw tentative translational conclusions.

As evidenced by the above review of the literature, a large variety of experimental approaches have been adopted in the study of pain-related cognitive impairment. These include differences in pain models and participant groups, differences in the methods of pain measurement and evaluation and differences in the cognitive outcome measures explored. The studies presented herein were intended to provide a more coherent investigation of pain-related cognitive impairment in both clinical and preclinical settings and the rationale for the experimental approaches taken is described below.

**Specific Aims:**

- To investigate cognitive function following SNL surgery in young and mid-aged rats
- To establish and validate a paradigm for the assessment of aversive learning and memory in rodent models of chronic neuropathic (SNL) and inflammatory (CFA) pain
- To determine whether pain-related impairment in cognitive flexibility is associated with alterations in the level of synaptophysin in the prefrontal cortex or hippocampus
• To assess the effects of chronic amitriptyline treatment on pain-related behaviour and cognitive function following SNL surgery in mid-aged rats

• To determine brain regional and spinal cord levels of monoamines and synaptophysin following SNL surgery in mid-aged rats and to investigate whether these levels are altered by administration of amitriptyline

• To determine whether a sample of chronic pain patients perform poorly compared with matched controls on a battery of cognitive tests and investigate the relative contribution of age and other potentially confounding factors

• To investigate whether cognitive performance in chronic pain patients is correlated with their pain scores.

1.5.1 Rationale for rodent pain models used

The SNL model of neuropathic pain was first introduced in 1992 by Kim and Chung, and has since been well validated and widely used. The surgical protocol involves tight ligation of one (L5) or two (L5 and L6) of the animal’s segmental spinal nerves. The surgery is performed unilaterally and results in neuropathic pain-related behaviour directed at the ipsilateral hindpaw compared to the contralateral hindpaw post surgery. The surgery is associated with long-lasting mechanical allodynia, cold allodynia, and thermal hyperalgesia. Significantly, the SNL model is a suitable model to use for behavioural studies examining the effects of neuropathic pain on cognition, as it is associated with minimal potentially confounding symptoms and illnesses. It is therefore preferable to models such as STZ-induced diabetic neuropathy, which is associated with hyperglycaemia, polyuria, reduced weight gain, and, in the case of high doses of STZ, significant weight loss and signs of liver and kidney toxicity. Furthermore, the surgery is not associated with significant motor deficits that may affect the cognitive outcomes (Chung et al., 2004). While SNL surgery results in robust evoked pain behaviours, it is not generally associated with spontaneous pain behaviour. As
such, we have also included investigations of cognitive behaviour following induction of inflammatory pain by subcutaneous intraplantar injection of CFA. This is generally considered to be a model of chronic pain analogous to highly clinically prevalent inflammatory conditions. Given the research outlined in Section 1.4, which suggests a role for age in the interaction between pain and cognition, we have also included mid-aged animals in a number of studies described.

1.5.2 Rationale for rodent behavioural cognitive tests used

A battery of established, well validated tests of cognitive behaviour was used to assess cognitive function in rat models of chronic pain. This battery included novel object recognition, T-maze and Morris water maze tasks. These tests measure predominantly spatial reference and recognition memory but may also be used to measure higher order functions such as cognitive flexibility (as in the case of spatial reversal in the water maze). These tests were selected as they are dependent on neural resources also required for pain processing. Thus, it was hypothesised that performance would be negatively affected in the presence of chronic pain. The relationship between pain and aversive learning and memory had been understudied, due in part to the lack of a suitable method of assessment. Thus, the development and validation of a novel air-puff-induced passive avoidance paradigm is outlined in Chapter 4.

1.5.3 Rationale for the investigation of synaptophysin and the monoaminergic system

A detailed experimental examination of all of the potential mechanisms involved in pain-related cognitive impairment was clearly beyond the scope of this dissertation. Therefore the experiments presented investigated a subset of neurochemical and neuroplastic mediators. As described in Section 1.2.3, synaptic plasticity is altered in models of chronic pain and in cognitive impairment. Pain-related plasticity is a major component of the theoretical model proposed to underlie the
mechanism of pain-related cognitive impairment. As such, the ability to quantify synapses in brain regions of relevance to pain and cognition was considered an important goal of the present research. The amount of synaptophysin is thought to correlate with the number of synaptic connections and so it is a useful tool in the assessment of alterations in synaptic plasticity.

The monoaminergic system is involved in both pain and cognition (see Section 1.2.4.7), but its involvement in pain-related cognitive impairment has not been well studied. In Chapter 6, we investigated whether there were pain-related alterations in monoamines in discrete brain regions which could in turn contribute to cognitive impairment. We also examined whether any changes in monoamines could be reversed with analgesic treatment.

1.5.4 Rationale for investigation of the effects of amitriptyline

The effects of oral administration of amitriptyline on pain and cognitive behaviours were investigated in Chapter 6. As outlined in Section 1.2.7, tricyclic antidepressants (TCAs) are commonly prescribed for the treatment of neuropathic pain in clinical situations and have also been shown to effectively reduce neuropathic pain-like behaviours in rodent models. A number of studies have shown that effective analgesia is capable of reversing pain-related effects on cognition. The antidepressant mechanism of action of amitriptyline is partly mediated by inhibition of the reuptake of the monoamines noradrenaline and serotonin. As discussed in Section 1.2.4.7, monoamines are implicated in the interaction between pain and cognition, and administration of amitriptyline in a model of neuropathic pain may therefore provide insights into the precise role played by the monoaminergic system. However, it should be noted that the analgesic mechanism of action of amitriptyline in neuropathic pain is not yet known, and this was also investigated.
1.5.5 **Rationale for selection of clinical participants and pain and cognitive measures**

The sample of chronic pain patients was drawn specifically from patients with a diagnosis of neuropathic pain or radiculopathy, so as to represent a relatively homogeneous group. The sample excluded people with a clinical diagnosis of depression or anxiety, which are common confounds in research on pain and cognition. Commonly used and well-validated scales were used for the assessment of pain, depressive symptoms and state-anxiety and similarly a battery of validated psychometric tests was used to evaluate aspects of cognition. The majority of the cognitive tests used were chosen to putatively correlate with the cognitive measures performed in rodents, and included tests of spatial and recognition memory and cognitive flexibility/executive function.
Chapter 2  Materials and Methods

This chapter and associated appendices describe the general materials and methods used in the studies that comprise this thesis. More detailed information pertaining to individual studies is provided in the “Materials and Methods” sections of subsequent results chapters.

2.1 Materials

2.1.1 Animal husbandry materials

Sprague-Dawley rats: Charles River (Margate, UK and L’Arbresle, France)
Rat cages: North Kent Plastics (Coalville, UK)
Water bottles: North Kent Plastics (Coalville, UK)
Goldflakes bedding: Wm. Lillico and Son (Horley, UK)
Rat chow: Harlan Teklad (Loughborough, UK)
Temperature/humidity monitor: Radionics Ltd. (Dublin, Ireland)
Weighing scales: Mason Technology (Dublin, Ireland)

2.1.2 Surgical procedure materials

Isoflo®: Abbott Laboratories (Maidenhead, UK)
Fucithalamic®: LEO Laboratories Ltd. (Dublin, Ireland)
Videne® iodine solution: Ecolab Ltd. (Leeds, UK)
Alcohol swabs: UHS (Enfield, UK)
Scalpel blades: Swann-Morton (Sheffield, UK)

Bulldog serrefines, retractor, small rongeur, hook, Michel 7.5 mm × 1.75 mm wound clips: Fine Science Tools, supplied by InterFocus Ltd. (Cambridge, UK)
CHAPTER 2. MATERIALS AND METHODS

Deknatel 6-0 silk suture: Teleflex Medical OEM (Galway, Ireland)
Vicryl Rapide 4-0 suture: Ethicon, Johnson & Johnson (Dublin, Ireland)
Baytril®: Bayer Healthcare (Dublin, Ireland)
Complete Freund’s adjuvant emulsifier: Sigma-Aldrich (Dublin, Ireland)
Syringes: BD Microlance (Oxford, UK)
Insulin syringes (29G × 12.7 mm): BD Microlance (Oxford, UK)
Needles (25G × 16 mm): BD Microlance (Oxford, UK)
Complete Freund’s adjuvant: Sigma-Aldrich (Dublin, Ireland)

2.1.3 In vivo testing equipment

Plethysmometer: Ugo Basile Srl. (Comerio, Italy)
von Frey arenas 1 and 3: constructed by Ambrose O’Halloran (Pharmacology and Therapeutics, NUI Galway)
von Frey arena 2: IITC Life Sciences Inc. (Woodland Hills, CA, USA)
Touch-Test® Sensory Evaluators: North Coast Medical Inc. (Gilroy, CA, USA)
Hargreaves arenas: IITC Life Sciences Inc. (Woodland Hills, CA, USA)
Plantar Analgesia Meter: IITC Life Science Inc. (Woodland Hills, CA, USA)
Cold allodynia arena 1: IITC Life Science Inc. (Woodland Hills, CA, USA)
Cold allodynia arena 2: constructed by Ambrose O’Halloran (Pharmacology and Therapeutics, NUI Galway)
Acetone: Sigma-Aldrich (Dublin, Ireland)
Novel object arenas: constructed by Ambrose O’Halloran (Pharmacology and Therapeutics, NUI Galway)
Batbox® Duet: Batbox Ltd. (Steyning, UK)
T-maze: constructed by Ambrose O’Halloran (Pharmacology and Therapeutics, NUI Galway)
Passive-avoidance arena: constructed by Ambrose O’Halloran (Pharmacology and Therapeutics, NUI Galway)
Ultrasound generator: ProSound™ 800, obtained from Maplin Electronics (Galway, Ireland)
Air-duster canisters GDP: Electrolube (Ashby de la Zouch, UK)
Morris water maze 1 – pool: ROM Plastics Ltd. (Galway, Ireland)
Morris water maze 1 – platform: constructed by Ambrose O’Halloran (Pharmacology and Therapeutics, NUI Galway)
Morris water maze 1 – enclosure and lighting: constructed and fitted by Ambrose O’Halloran (Pharmacology and Therapeutics, NUI Galway)
White poster paint: Icon Apprentice, obtained from Cregal Art (Galway, Ireland)
Morris water maze 2 – pool: Engineering & Design Plastics Ltd. (Cambridge, UK)
Morris water maze 2 – platform: HVA Image Labs (Buckingham, UK)
Morris water maze 2 – lighting: Tim Kelly Electrical Contractors Ltd. (Ballinrobe, Ireland)
Video cameras: Nikon/G|AZNZ, supplied by Radionics Ltd. (Dublin, Ireland)
DVD recorders: Sony, obtained from Currys (Galway, Ireland)
DVD+R: Tesco (Galway, Ireland)

2.1.4 Drugs and drug administration

Distilled H$_2$O
Saline (0.89% NaCl): Sigma-Aldrich (Dublin, Ireland)
Scopolamine hydrobromide: Sigma-Aldrich (Dublin, Ireland)
Amitriptyline hydrochloride: Sigma-Aldrich (Dublin, Ireland)
Syringes: BD Microlance (Oxford, UK)
Needles (25G × 16 mm), BD Microlance (Oxford, UK)

2.1.5 Transcardial perfusion

Heparin (5000 U/ml): Wockhardt UK Ltd. (Wrexham, UK)
Sodium dihydrogen phosphate monohydrate: Applichem, supplied by Lennox (Dublin, Ireland)
Disodium hydrogen phosphate dehydrate: Applichem, supplied by Lennox (Dublin, Ireland)
Paraformaldehyde: Sigma-Aldrich (Dublin, Ireland)
Phosphate-buffered saline: Sigma-Aldrich (Dublin, Ireland)
CHAPTER 2. MATERIALS AND METHODS

Sucrose: Lennox (Dublin, Ireland)
Sodium azide: Sigma-Aldrich (Dublin, Ireland)

2.1.6 Histology and immunohistochemistry

Microm cryostat: Thermo Fisher Scientific (Walldorf, Germany)
Superfrost®-charged microscope slides: Fisher Scientific (Dublin, Ireland)
Stoppered Grenier pots: Cruinn Diagnostics (Dublin, Ireland)
FITC-conjugated Griffonia (Bandeiraea) Simplicifolia Lectin 1, IB4: Vector Laboratories (Peterborough, UK)
Gelmount™: Sigma-Aldrich (Dublin, Ireland)
Fluoromount™: Sigma-Aldrich (Dublin, Ireland)
Normal rabbit serum: Sigma-Aldrich (Dublin, Ireland)
Triton-X: Sigma-Aldrich (Dublin, Ireland)
Monoclonal anti-synaptophysin: Millipore Ireland BV (Carrigtwohill, Ireland)
AF488-conjugated rabbit anti-mouse IgG: Invitrogen, supplied by Bio-Sciences Ltd. (Dun Laoghaire, Ireland)
Coverslips: Fisher Scientific (Dublin, Ireland)
Inverted microscope with structured light illumination: Olympus (Department of Anatomy, NUI Galway)
FluoView™ 300 confocal microscope: Olympus (Department of Anatomy, NUI Galway)

2.1.7 HPLC

Distilled H₂O, resistivity above 16.7 MΩ.cm
Citric acid monohydrate: Sigma-Aldrich (Dublin, Ireland)
Sodium dihydrogen phosphate monohydrate: Applichem, supplied by Lennox (Dublin, Ireland)
1-octane sulphonic acid sodium salt: Sigma-Aldrich (Dublin, Ireland)
Ethylenediaminetetraacetic acid disodium salt dehydrate: Sigma-Aldrich (Dublin, Ireland)
CHAPTER 2. MATERIALS AND METHODS

Methanol 215: Romil, supplied by Lennox (Dublin, Ireland)
NaOH: Sigma-Aldrich (Dublin, Ireland)
Reverse phase analytical column (Licro-sorb c18, length 250 cm and internal diameter 4.0 mm): Phenomenex (Macclesfield, UK)
Electrochemical detector: Shimadzu (Milton Keynes, UK)
Merck-Hitachi D-2000 integrator: Agilent Technologies (Dublin, Ireland)

2.1.8 Western blotting

SDS, Trizma®-Base, HCl, glycerol, bromophenol blue, β-mercaptoethanol, sodium β-glycerophosphate, dithiothreitol, sodium fluoride, protease-inhibitor cocktail, ammonium persulphate, glycine, Tween 20, acrylamide mix, TEMED: Sigma-Aldrich (Dublin, Ireland)
Milk formula: Aptamil™, obtained from Tesco (Galway, Ireland)
Minigel electrophoresis system, plates, combs, gaskets, clamps, Fisherbrand power pack: Fisher Scientific (Dublin, Ireland)

2.1.9 Clinical study materials

Chronic Pain Grade questionnaire: obtained from Test Library, School of Psychology, NUI Galway
Patient Health Questionnaire: ©Pfizer Inc., obtained from Test Library, School of Psychology, NUI Galway
Spielberger State Anxiety Inventory (Form Y): obtained from Test Library, School of Psychology, NUI Galway)
WAIS®-III: Psychological Corporation, obtained from Test Library, School of Psychology, NUI Galway
WMS-III: Psychological Corporation, obtained from Test Library, School of Psychology, NUI Galway
Continuous Performance Task: MATRICS Assessment Inc., obtained from Department of Psychiatry, NUI Galway
Wisconsin Card Sorting Task – CV4: PAR Inc., supplied by Brainworx (Avoca, Ireland)
CHAPTER 2. MATERIALS AND METHODS

2.1.10 Computer software

Microsoft® Office: Microsoft Ireland (Dublin, Ireland)
SPSS/PASW (versions 15–18): SPSS Inc. (Chicago, IL, USA)
GraphPad Prism® Version 5: GraphPad Software Inc. (La Jolla, CA, USA)
Ethovision® 3.1: Noldus (Wageningen, The Netherlands)
Ethovision® XT 7: Noldus (Wageningen, The Netherlands)
Volocity® 3D image analysis software: PerkinElmer (Waltham, MA, USA)
GeneSnap image acquisition software: Syngene (Cambridge, UK)
ImageJ software: National Institute of Health (Bethesda, MD, USA)
ImageJ software: McMaster Biophotonics Facility (Hamilton, ON, Canada)
HP Chemstations for LC and LC/MS Systems: Agilent Technologies (Dublin, Ireland)

2.2 Methods

2.2.1 Animal experimental subjects

Experimental procedures on animal subjects were conducted with the approval of the Animal Care and Research Ethics Committee of the National University of Ireland, Galway, under licence from the Irish Department of Health and Children and in compliance with European Communities Council directive 86/609 and International Association for the Study of Pain (IASP) guidelines. Male Sprague-Dawley rats were used for all of the experiments described. Young adults of the CD strain (used in results Chapters 3 and 4) were obtained from Charles River (Margate, UK) and weighed 140–300 g (approximately 4–9 weeks old) on arrival. Mid-aged (9 months old) adults of the OFA strain (used in results Chapters 5 and 6) were obtained from Charles River (L’Arbresle, France) and weighed 465–675 g on arrival. The large range in weights for these rats is due to the fact that they were supplied according to age rather than weight. In all cases, animals were randomly assigned to treatment groups, and there were no differences in
average weight between the groups within each experiment. Rats were housed individually in opaque plastic-bottomed cages (50.5 cm × 24 cm × 13 cm: length × width × height) with a wire grid top. Bedding consisted of wood shavings, changed weekly. Rats were maintained under standard laboratory conditions of temperature (20 ± 2)°C, humidity (40–60%) and lighting (12:12 hour light/dark cycle, lights on at 08:00). Standard rat chow pellets (Harlan Laboratories UK Ltd., Loughborough, UK) and water were available ad libitum.

2.2.2 Animal models of chronic pain

2.2.2.1 Spinal nerve ligation surgery

Spinal nerve ligation (SNL) surgery, originally described by Kim and Chung (1992), was used to model chronic neuropathic pain. The surgery involves ligation of the lumbar spinal nerves L5 and L6. Rats were anaesthetized with isoflurane (Isoflo®, Abbott Laboratories, Maidenhead, UK, 5% in 0.6 l/min oxygen for induction and 2.5% in 0.6 l/min oxygen for maintenance). A drop of 1% fucidic acid viscous eye drop preparation (Fucithalamic®, LEO Laboratories Ltd., Dublin, Ireland) was placed on each eye to prevent them from drying out. The fur lateral to the midline on the left-hand side at the lower lumbar and sacral regions was clipped closely. The area was cleaned with an ethanol swab (UHS, Enfield, UK) and treated with antiseptic iodine solution (7.5% w/w, iodinated povidone, Videne®, Ecolab Ltd., Leeds, UK). An incision was made using a sterile scalpel blade (no. 21, Swann-Morton®, Sheffield, UK) through the skin between the spinal column and the left iliac crest. Bulldog serrefines (Fine Science Tools, cat. no. 18050-28, supplied by InterFocus Ltd., Cambridge, UK) were used to clamp the skin back and a retractor (Fine Science Tools, cat. no. 17005-04) was used to open the surgical cavity. A diamond-shaped piece of muscle tissue was excised with a scalp blade and scissors, and the paraspinal muscles were removed using a toothed forceps to visualise the L6 transverse process. This bone was then removed using a small rongeur (Fine Science Tools, cat. no. 16021-14), exposing
the L4 and L5 spinal nerves (see Figure 2.1). 6-0 silk suture (0.07 mm diameter, Deknatel®, Teleflex Medical OEM, Galway, Ireland) was looped around the L5 nerve using a pair of fine curved forceps and the nerve was tightly ligated. The L6 nerve, which is located underneath the sacrum, was isolated using a small hook (Fine Science Tools, cat. no. 1006-12) and was also ligated. Care was taken to avoid any damage to the L4 spinal nerve which is lateral to L5 and provides motor innervation to the hindpaw. In the sham-operated rats, the L5 and L6 nerves were exposed but were not ligated. Haemostasis was confirmed before closing the wound. For both sham and spinal nerve ligated rats, the wound was sutured internally (Vicryl Rapide 4-0, Ethicon, Johnson & Johnson, Dublin, Ireland) and the skin was closed with sterile wound clips (Michel, 7.5 mm × 1.75 mm). Rats were injected intraperitoneally (i.p.) with 1 ml sterile saline to replace fluid lost during surgery. Baytril® (stock solution 25 mg/ml enrofloxacin, Bayer Healthcare, Dublin, Ireland) was diluted 1:10 and was also injected i.p. at a dose of 2.5 mg/kg in a volume of 1 ml/kg. The total surgery duration was approximately 1.5 hours. Rats were allowed to recover from anaesthesia in recovery cages maintained at a constant temperature on a heating pad and then rehoused singly with fresh bedding in their home cages. Animal health was closely monitored in the 24 hours post surgery, and on a daily basis thereafter.

### 2.2.2.2 Complete Freund’s adjuvant injection

Immunogenic complete Freund’s adjuvant emulsifier (CFA, desiccated Mycobacterium tuberculosis in an 85% mineral oil, 15% mannide monooleate suspension, Sigma-Aldrich, Dublin, Ireland) was used to induce a chronic inflammatory pain state (Stein et al., 1988). Rats received a single 100 µl intraplantar injection of CFA (1 mg/ml) into the left hindpaw, under brief isoflurane anaesthesia (5% in 0.8 l/min oxygen). Control rats underwent intraplantar needle insertion to the left hindpaw, also under isoflurane anaesthesia. After a brief recovery period, rats were rehoused singly in their home cages.
Figure 2.1: Left: Schematic representation of L5/L6 spinal nerve ligation surgery in the rat (from Chung et al., 2004). Right: Representative photographic image of ligated rat L5 and L6 spinal nerves.
2.2.3 Physiological and behavioural characteristics of chronic pain models

2.2.3.1 Measurement of hindpaw oedema

Paw circumference and paw volume were measured under isoflurane anaesthesia (5% in 0.8 l/min oxygen) immediately before CFA injection and again 21 days later. Circumference was measured by wrapping a piece of string around the widest part of the left hindpaw and measuring the length of the string. Paw volume was measured using a plethysmometer (Ugo Basile Srl., Comerio, Italy) – see Figure 2.2. The reservoir was filled with a 0.5% Teepol (Lennox Laboratory Supplies, Dublin, Ireland), 0.05% NaCl (Sigma-Aldrich, Dublin, Ireland) solution. The left hindpaw was submerged into the measuring chamber up to the level of the lateral malleolus (see Figure 2.3) which was marked with a permanent marker, and the paw volume was measured by displacement. Paw volume was shown on the display unit, and the average of three separate sequential recordings was calculated. The reservoir was replenished between rats. The experimenter performing the measurements was blind to the treatment, though there may have been visible oedema at the post-injection measurement time point (3 weeks post injection). The resolution of the instrument was 0.01 ml, and the observed variations between the three measurements for each rat were 0.03–0.31 ml (i.e., < 10% of the average paw volume).

2.2.3.2 Behavioural measurement of sensory responses

Successful SNL surgery or induction of chronic inflammatory pain by CFA injection results in several behavioural outcomes indicative of chronic pain, including mechanical and cold allodynia, and heat hyperalgesia in the injured (ipsilateral) paw. In this section, we describe the test procedures used to measure these behavioural responses.

von Frey test for mechanical allodynia

The arena used for assessment of mechanical allodynia differed for different exper-
Figure 2.2: Plethysmometer apparatus set-up (adapted from Ugo Basile Plethysmometer Instruction Manual, 1996).

Figure 2.3: Photograph indicating the position of the lateral malleolus of the rat hindpaw.
iments. In general, consisted of a six-chambered arena made of clear Perspex or white, melamine-coated chipboard (illustrated schematically in Figure 2.4). The dimensions of the chambers were such that rats could move freely. Three different arenas were used over the course of the studies described herein. Their dimensions were: (1) 16 cm (length, \(l\)) \(\times\) 20 cm (width, \(w\)) \(\times\) 43 cm (height, \(h\)), (2) 11 cm \(l\) \(\times\) 20 cm \(w\) \(\times\) 15 cm \(h\) and (3) 14 cm \(l\) \(\times\) 20 cm \(w\) \(\times\) 25 cm \(h\). Arena 1 was used in the experiment described in Chapter 3, arena 2 in those described in Chapter 4 and arena 3 for the experiments of Chapters 5 and 6. Changes in arena sizes were made due to variability in the weight ranges of rats used and other technical considerations (cost, availability and behavioural tracking). A Perspex lid with air-holes was placed on top of the arena during the habituation and testing periods. In all experiments, the arena was placed on a raised wire-mesh flooring so that the experimenter could access the rats' hindpaws from below (see Figure 2.4). Rats received an initial habituation period of 30 minutes during which they were placed in individual chambers of the arena. No testing was carried out, and at the end of the 30-minute period the rats were returned to their home cages. On subsequent test days, the rats were habituated to the arena for an additional 20 minutes prior to assessment. In most studies, baseline testing was carried out prior to surgery. The method used was adapted from several test protocols described previously (Chaplan et al., 1994; Kim and Chung, 1992; Tal and Bennett, 1993), and was such that both the 50% withdrawal threshold and the % response to individual filaments could be quantified. von Frey filaments (Touch-Test® Sensory Evaluators, North Coast Medical Inc., Gilroy, CA, USA) of different weights (0.07 g – 180 g) were used, starting with filament number 10 (2 g). The filament was applied perpendicular to the plantar surface of the hindpaw, targeting the area at the base of the third and fourth digits (shown previously to be the most sensitive area of the paw after SNL, Chung et al., 2004), with sufficient force to cause slight buckling of the filament, for approximately 6 seconds or until a positive result was observed. A positive result was recorded if
flinching, licking or withdrawal of the paw occurred on application of the filament or immediately after removal of the filament. Filaments were applied to both left and right hindpaws five times (alternating between paws). If a positive response was observed to any of the five applications using the 2 g filament, filaments of lower weights were applied in descending order until no positive responses were observed. Filaments were then applied in ascending order of weight until positive responses to five applications were observed on two consecutive filaments. Six rats were tested per session, and the arena was thoroughly cleaned between each session using a mild detergent solution.

The % percentage response to individual filaments was calculated using the formula:

\[
\left( \frac{\text{number of responses}}{5} \right) \times 100
\]

The filament weight eliciting 50% response was calculated by plotting a non-linear regression curve of the % response versus filament weight for each rat (using GraphPad Prism® software).

**Hargreaves test for thermal hyperalgesia**

The method used for the assessment of thermal hyperalgesia was similar to that described by Hargreaves et al. (1988), and was performed using a commercially available apparatus (Plantar Analgesia Meter, IITC Life Science Inc., Woodland Hills, CA, USA). The apparatus consisted of a six- or three-chambered Perspex arena placed on top of a raised, heated, glass plate (30 ± 1)°C, as shown in Figure 2.5. For the six-chambered arena, each chamber was 11 cm (l) × 20 cm (w) × 15 cm (h); for the three-chambered arena, chambers were 22 cm (l) × 20 cm (w) × 15 cm (h). Each chamber was separated from the adjoining one by a Perspex partition. Rats were placed in individual chambers and underwent an initial 30-minute habituation period to the test apparatus. On test days, rats were habituated to the arena for 20 minutes prior to assessment. A moveable radiant heat source was positioned underneath the glass and could be focused on
Figure 2.4: von Frey mechanical allodynia testing. (a) Schematic illustration of the apparatus used. (b) Photograph of the testing procedure. (c) Plantar surface of rat hindpaw, showing the area (shaded) at which the von Frey filament was targeted.
the rat’s hindpaw using the “guide light” (idle intensity 1%) and the adjustable, angled mirror on the heat source (see Figure 2.5). The heat source was set to an active intensity of 30% and the stimulus was applied from below to the plantar surface of the rat’s hindpaw (at the same stimulus location as for the von Frey filaments) until a positive response (criteria similar to those used for von Frey testing as described earlier in this section) was recorded or until a cut-off time of 20 seconds was reached. This cut-off time was selected to prevent tissue damage. If the animal did not exhibit a positive response within the cut-off time, a latency of 20 seconds was recorded. The initialisation of the trial was controlled by the experimenter using a pushbutton, and the response latency was recorded automatically. Right and left hindpaws were each tested four times, alternating between paws. Rats were tested in groups of six per session, and the arena was thoroughly cleaned between each session using a mild detergent solution. The average withdrawal latency for each paw over the four trials was calculated.

Acetone drop test for cold allodynia

The apparatus used for cold allodynia testing was similar to that used for von Frey testing, consisting of a six-chambered arena constructed from clear Perspex or white melamine coated chipboard, placed above a raised, wire-mesh flooring. The test protocol was adapted from that of Choi et al. (1994). Rats were habituated to the test apparatus in a similar way to the other sensory tests. A short length of polyethylene Portex® (Fisher Scientific, Dublin, Ireland) tubing (2 mm internal diameter, 3 mm external diameter) was attached to a 1 ml syringe. This was used to apply 0.2 ml of acetone (Sigma-Aldrich, Dublin, Ireland) to the plantar surface of the hindpaw (at the same stimulus location as for the von Frey filaments), without stimulating the paw mechanically. Surface tension causes the drop to spread across the skin and evaporation causes a cooling sensation. Each hindpaw was tested three times, alternating between paws. Rats were tested in groups of six per session and the arena was thoroughly cleaned between each session using
Figure 2.5: Hargreaves test: (a) apparatus set-up (b) delivery of test.
a mild detergent solution. A stopwatch was used to record the latency to the first paw withdrawal, and the number of positive responses (with criteria similar to those used for von Frey testing as described above) within 60 seconds of acetone application was counted for each trial. The average latency to the first response and the total number of withdrawals across trials was then calculated.

2.2.4 Behavioural measurements of cognitive function

2.2.4.1 Novel object recognition test

Novel object recognition is based on the natural tendency of the animal to spend more time exploring new, rather than previously encountered objects (Berlyne, 1950), and was first described as a measure of recognition memory by Ennaceur and Delacour (1988). The procedure used herein was based on a number of protocols described previously (Bevins and Besheer, 2006; King et al., 2004), with some modifications. Testing was carried out in one of two specially constructed arenas: a rectangular arena, made from black melamine-coated chipboard with a clear Perspex base, with dimensions 60 cm (l) × 30 cm (w) × 40 cm (h) (Chapter 3); or a circular arena, with a wooden base (painted black) and metal sides (also painted black), with dimensions 75 cm (diameter, D) × 38 cm (h) (Chapter 4). Both arenas are represented schematically in Figure 2.6. In all experiments, the arena was illuminated by four 60 W bulbs which provided constant light intensity of (150 ± 10) lux (Chapter 3) or (100 ± 20) lux (Chapters 4, 5 and 6) at floor level of the arena. A camera (Nikon®, positioned above the arena fed live images to a DVD recorder (Sony®, Model no.: RDR-GX350) for subsequent analysis. A bat detector (Batbox Duet, Batbox® Ltd., Steyning, UK) was placed adjacent to the arena to determine whether rats emitted any fear-associated (22 kHz) ultrasonic vocalisations. No vocalisations in the aversive range were detected on the test day in any of the novel object recognition experiments (data not shown), suggesting that both the arena and the objects presented were perceived as neutral. The objects used included plastic bottles, covered with white masking tape and filled
with water, with a base diameter of 6.5 cm and 18.5 cm height, plastic Coca-Cola® bottles with a base diameter of 4.5 cm and 23.5 cm height, a glass bottle of base diameter 5 cm and height 10.3 cm, filled with red coloured liquid, and an abstract plastic structure with base 5 cm × 5 cm and height 16cm constructed from green and blue toy blocks (Playskool Clipo™ blocks). In all cases, the objects had no apparent natural significance to the rats, and were secured to the base of the arena with UHU® white tack such that they were difficult to displace.

Animals were habituated to the arena in the absence of objects for 20–30 minutes on the day before test day.

The test day comprised three stages: (i) Habituation (ii) Exposure 1 (iii) Exposure 2. Rats were introduced to the arena for a 3-minute habituation period on the test day and then returned to their home cage for 7 minutes. During Exposure 1, two identical objects were placed in the arena, either 12.5 cm from side and end-walls in opposing corners of the rectangular arena, or 16 cm from points on the perimeter of the circular arena (see Figure 2.6). The rat was allowed to freely explore the arena and objects for a period of 3 minutes, after which the animal was removed from the arena and returned to its home cage for an interval of between 2 and 5 minutes. A 2-minute inter-exposure interval was used in Chapter 3, while a 5-minute interval was used in the experiments described in Chapters 5 and 6. Prior to Exposure 2, one of the objects was replaced with a novel object. The animal was again allowed to freely explore the arena and objects for a period of 3 minutes and then returned to its home cage. Representative images from Exposure 1 and Exposure 2 are shown in Figure 2.6. The arena was cleaned with a mild detergent between rats to remove odours and olfactory cues, and faecal pellets were removed between exposures. The total number of faecal pellets in each exposure was also recorded as a measure of anxiety-related behaviour. There were no differences in the faecal count between sham and SNL rats or between exposures in any of the experiments (data not shown).

Exploration of an object was defined as sniffing the object, rearing against
Figure 2.6: (a) and (b) Schematic diagrams of the two arenas used for novel object recognition testing. (c) and (d) Representative images from Exposures 1 and 2, respectively, on the novel object recognition test day.
the object or having the head directed towards the object within a 2 cm annulus of the object. Exploratory behaviour and general behaviours of sniffing, rearing, grooming, and freezing were manually rated from the DVD recordings of each of the three test stages, with the aid of Ethovision® behavioural tracking software version 3.1 or version XT7 (Noldus Information Technology, Wageningen, The Netherlands). In all cases the experimenter rating the behaviour was blind to the experimental treatment of the rat (surgery or drug). The software was also used to track the distance (in cm) moved by the animal during testing. Procedures for use of the Ethovision® system are outlined in detail in Appendix C. The proportion of time spent exploring the object was assessed by calculating a discrimination ratio as follows:

\[
\frac{\text{total time spent exploring either object}}{\text{total time spent exploring both objects}}
\]

The position of the novel object was alternated between rats such that it was on the right-hand side of the arena for 50% of the animals and on the left for the other 50%. This was in order to minimise potential confounding effects related to orientational biases.

### 2.2.4.2 T-maze spontaneous alternation

T-maze spontaneous alternation is used to measure exploratory behaviour and spatial memory, and is based on rodents’ tendency to explore novel environments, i.e., they prefer to visit a new arm of the maze rather than a familiar arm (Dennis, 1939). The procedure for T-maze testing was similar to that described by Deacon and Rawlins (2006), with some minor modifications. Testing was carried out in a specially constructed T-maze of height 40 cm, made from wood and painted black (see Figure 2.7 for additional dimensions). The guillotine doors and central partition were also constructed from wood and painted black. A camera was positioned above the T-maze. Live images were recorded to DVD for subsequent analysis with Ethovision® behavioural tracking software (see Appendix C). The maze was placed in a dimly lit room (16–20 lux at floor level of maze). The maze
was set up with the central partition in place and guillotine doors removed. Rats were placed in the start arm and allowed to choose a goal arm. Once a goal arm was chosen the rat was confined to that arm for a period of 30 seconds by lowering the guillotine door. The central partition was then removed, and the rat was returned to the start arm and again allowed to choose a goal arm. The criterion for goal arm selection was that the entire animal (including the tail tip) be on the goal arm, and a cut-off time of 90 seconds was selected. If the rat failed to select an arm within 90 seconds, it was removed from the maze and this was considered an error of omission. Rats received ten trials in total (five trials per day for two days) and the mean % alternation was calculated for each animal. The average velocity (in cm/s) during trials and the latency (in seconds) to choose an arm were also recorded. Velocity (rather than total distance moved) was used as the readout of locomotor activity in this case, because the duration of the trials was not fixed.

Figure 2.7: Schematic representation of the apparatus used for testing T-maze spontaneous alternation.
2.2.4.3 Morris water maze

The Morris water maze (MWM), first described by Morris (1984), is an established method used to assess spatial learning and memory, and allows measurement of a number of different cognitive parameters using distinct tests (Vorhees and Williams, 2006).

Apparatus

Two different arenas were used for different experiments. The first, used for the experiments described in Chapter 3, consisted of a circular black plastic pool 95 cm in diameter, with a weighted platform made of white plastic measuring 11 cm ($w$) × 43 cm ($h$). The second, used for the experiments described in Chapters 5 and 6, consisted of a circular white plastic pool 2 m in diameter, and the platform used in this case was made of clear Perspex with dimensions 10 cm ($w$) × 30 cm ($h$). An image of the apparatus is shown in Figure 2.8. The maze was filled with water, such that the water level was 2 cm less than the height of the platform for cued testing and 2 cm greater than the height of the platform for acquisition and reversal training. During acquisition and reversal, the platform was submerged and was invisible to the rat. The water in the black arena was made opaque by addition of 200 ml white non-toxic water-based poster paint (Icon Apprentice), so as to obscure the rats’ view of the platform. The lighting of the arena was kept constant at (100 ± 20) lux (at water level) and the water temperature was maintained at (25 ± 3)°C throughout the testing. A video camera located above the apparatus was connected to a DVD recorder for subsequent behavioural tracking or output directly to the Ethovision® system (see Appendix C). The maze was surrounded by curtains, on which visual cues were hung during acquisition and reversal training and during probe trials. The cues consisted of geometric shapes (a filled circle, an open square and a series of wavy lines) printed in black on white A4 or A3 paper.
Figure 2.8: (a) Schematic of the apparatus used for Morris water maze testing. (b) Photograph showing a Morris water maze acquisition trial. The position of the hidden platform is indicated by the dashed red circle.
Table 2.1: Trial pattern for the Morris water maze cued-learning test. The first position denotes the release point and the second position denotes the platform quadrant. N = North, S = South, E = East, W = West, SE = Southeast, NE = Northeast, SW = Southwest. Adapted from Vorhees and Williams (2006).

Cued test

We used the cued test as a control procedure to assess the rats’ vision, ability to swim, and ability to identify the platform as an escape route from the maze. For this test, the pool was filled to a level of 2 cm below the height of the platform, and the top of the platform was covered with black plastic so that it was clearly visible in the water. The visual cues were not used for this part of testing. Animals were released from one of the four start positions in a quasi-random order (see Table 2.1) and the platform was moved between trials. The animals were given 120 seconds to locate the platform, and having located it they were allowed to remain on it for 10 seconds. Latency to find the platform and path length (in cm) from release position to platform were recorded. It is expected that if vision is intact, the rat will swim directly to the visible platform and that the latency to get on to the platform will decrease over the four trials.

Acquisition training

The acquisition phase is used to investigate the rats’ ability to learn the position of the hidden platform by reference to spatial cues external to the maze. The pool was filled to 2 cm above the level of the platform such that the platform was hidden below the surface. The cues were hung on the curtains surrounding the pool and were visible at water level. The training consisted of four trials per day over five consecutive days, throughout which the platform remained positioned in the southwest quadrant of the pool. For each trial, the animal was released...
Table 2.2: Release positions for Morris Water Maze acquisition training trials. The sequence of release positions is designed such that the platform (which is located in the SW quadrant) will be to the right or left of the animal during an equal number of trials and that one trial will occur from each of the four start positions each day. N=North, S=South, E=East, SE=Southeast, NE=Northeast, SW=Southwest. Adapted from Vorhees and Williams (2006).

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with its head facing towards the wall of the pool and away from the platform. The rat was released from one of the four release points (Table 2.2) and was given 120 seconds to locate the hidden platform. Once the animal located the platform successfully, it was allowed to remain there for 10 seconds. If the animal did not locate the platform within 120 seconds, it was guided towards it by the experimenter and placed onto the platform for the 10 seconds. The animal was then removed from the pool, dried off with a cotton towel and returned to a heated recovery cage for an inter-trial period (approximately 5–15 minutes) before the next trial. The order in which the rats were tested was randomised every day to avoid any confounding factors that may have been present due to the time of day at which testing was performed.

Probe trial

The probe trial tests the rats’ reference memory of the platform location. The probe trial was carried out the day after acquisition training was completed. The platform was removed from the maze, and the rats were all released from the northeast quadrant. The probe trial lasted 120 seconds and consisted of only one trial per rat. The proportion of time the rat spent in each quadrant was
determined with the aid of Ethovision® software (see Appendix C). It is expected that rats should spend a larger proportion of time in the quadrant where the platform had previously been located (southwest).

Spatial reversal
The spatial reversal task is termed a measure of “cognitive flexibility”, and requires both extinction of the learned response and further spatial learning of a new platform location. The protocol for testing was the same as for acquisition training except that the platform was positioned in the opposite quadrant of the pool (northeast). Reversal testing was performed over five consecutive days. The parameters tested were: the proportion of the time spent and the proportion of the distance moved in the “old” location or area where the platform had been located (southwest quadrant), and the proportion of the time spent and the proportion of the distance moved in the “new” platform location (northeast quadrant). At the end of reversal training, a reversal probe trial was carried out. This was similar to the initial probe trial except that the rats were all released from the southwest quadrant.

2.2.4.4 Passive avoidance
Passive-avoidance paradigms are used to measure context-conditioned aversive learning and memory. A mild foot-shock is the most commonly used aversive stimulus in such paradigms. However, as we aimed to investigate aversive learning and memory in models of chronic pain (which are associated with hypersensitivity of the hindpaws), we examined alternative aversive stimuli as described below.

Ultrasound
A rat passive-avoidance paradigm employing ultrasound as the unconditioned aversive stimulus has been described previously (Dokla et al., 1989). We used a similar test protocol, with some modifications. Passive-avoidance testing was carried out in a specially constructed light/dark arena. The light compartment
was made of white melamine-coated chipboard with dimensions of 30 cm (l) × 30 cm (w) × 40 cm (h), and was lit from above using a standard 60 W bulb such that the compartment was maintained at a constant light intensity of (100 ± 10) lux. The dark compartment was made of dark grey Perspex and its dimensions were identical to those of the light compartment. The dark compartment was also fitted with a lid made of black wood to minimise the entry of light to the compartment, and light intensity in the compartment was negligible. The two compartments were separated by a manually-controlled guillotine door. Video cameras were fitted above the light compartment and in the lid of the dark compartment to monitor rat behaviour, and live images were recorded to DVD. Testing consisted of an acquisition trial in which the rat was placed in the light compartment of the arena, facing away from the guillotine door, and the latency to enter the dark compartment was recorded up to a maximum of 300 seconds. If the rat did not enter the dark chamber within the 300-second period, it was removed from the arena, returned to its home cage and excluded from further testing. Once the rat had entered the dark compartment, the guillotine door was lowered by the experimenter. After a lag time of 30 seconds, ultrasound at an aversive frequency of 21 kHz and a volume of (100 ± 10) dB was delivered for a period of 1 minute through a speaker fixed in the lid of the dark compartment. The rat was then removed from the arena and returned to its home cage. Control rats went through an identical procedure to that described above, but were not exposed to ultrasound. The retention trial was carried out 24 hours after the acquisition trial. The rat was again placed in the light compartment of the arena, facing away from the guillotine door, and the step-through latency to enter the dark compartment was recorded. If the animal did not enter the dark compartment within 300 seconds, it was removed from the arena and returned to its home cage, and latency was recorded as 300 seconds. No ultrasound was delivered during retention trials. The arena was cleaned with mild detergent between rats to remove any olfactory cues present.
Air-puff
We also used air-puff as an aversive stimulus, and as an alternative to foot-shock, in an attempt to induce a passive-avoidance response. The apparatus was identical to that described for the ultrasound investigation, but was not connected to the ultrasound generator. For delivery of air-puff directed at the rat’s face, an air-duster canister (GDP, Electrolube, Ashby de la Zouch, UK) was mounted to the outside of the dark compartment, such that a nozzle could protrude up to 3.5 cm into the dark compartment through a 5mm-diameter hole in the compartment wall opposite the guillotine door. The nozzle was positioned 3 cm from the floor of the arena (approximately nose-height of the rat). The vapour pressure of the compressed air in the air-duster canister used to deliver the air-puff was 62.8 kPa at 20°C. In the acquisition trial, the rat was placed in the light compartment of the arena facing away from the guillotine door, and the latency to enter the dark compartment was recorded up to a maximum of 300 seconds. Once the rat had entered the dark compartment, the guillotine door was lowered by the experimenter. Once the rat was facing the wall opposite the guillotine door, a single, brief puff of air (duration of ~1 second) was administered to the face. The rat was confined to the dark compartment for a further 90 seconds post air-puff, after which it was removed from the arena and returned to its home cage. If the rat did not enter the dark chamber within the 300-second period, it was removed from the arena, returned to its home cage and excluded from further testing. Control rats went through an identical procedure to that described above, but were not exposed to air-puff. For the retention trial, carried out 24 hours post acquisition, the rat was again placed back into the light compartment of the arena, facing away from the guillotine door, and the step-through latency to enter the dark compartment was recorded. If the animal did not enter the dark compartment within 300 seconds, it was removed from the arena and returned to its home cage, and latency was recorded as 300 seconds. No air-puff was administered during the retention trial, and the arena was cleaned with mild detergent between rats to
remove olfactory cues. In addition to the acquisition and retention trials, a 24-hour pre-test habituation period was also included in the air-puff passive-avoidance paradigm. During this period, rats were placed in the light compartment of the arena, facing away from the guillotine door, and allowed to freely explore both the light and dark compartments of the arena. After this period, the rat was removed from the arena and returned to its home cage. The purpose of the habituation period was to minimise anxiety induced by exposure to a novel arena.

As a similar air-puff-induced passive-avoidance paradigm had not been described previously, a detailed account of the method development and pharmacological validation is provided in Chapter 5.

2.2.5 Drugs

Scopolamine hydrobromide and amitriptyline hydrochloride were obtained from Sigma-Aldrich, Dublin, Ireland. Scopolamine was administered systemically by i.p. injection and was freshly prepared on the morning of use by dissolving in 0.9% sterile saline to the required concentration (1 mg/kg or 3 mg/kg in a volume of 1 ml/kg). Scopolamine was administered 30 minutes before acquisition in the passive-avoidance paradigm (see Chapter 4). The dose, route and time of administration were based on previous studies showing scopolamine-induced deficits in similar behavioural paradigms (Chopin et al., 2002; Park et al., 2002; Rosi et al., 2004; Sarter and Bruno, 1997; Steele et al., 1997). Amitriptyline was administered orally, in rats’ drinking water. Amitriptyline was dissolved in tap water, at a concentration of 0.3 g/l, and a fresh stock was prepared every 2 days. 150 ml of the stock solution was added to individual opaque water bottles, and the bottles were weighed daily to monitor consumption and to calculate the dose that each rat received. Based on the monitored fluid consumption and the weight of the rats, the expected dose of amitriptyline was between 8 and 18 mg/kg/day, in line with previous studies in which amitriptyline was administered in the drinking water (Esser et al., 2001; Yau et al., 2002).
CHAPTER 2. MATERIALS AND METHODS

2.2.6 Animal sacrifice and tissue collection

Transcardial perfusion and decapitation were used as humane experimental endpoints.

2.2.6.1 Transcardial perfusion

Rats were terminally anaesthetised with 5% isoflurane in 0.6 l/min oxygen (Isoflo®) until the heart rate slowed. The rat was then placed in a supine position and an incision was made in the abdomen, just below the ribcage, using a sharp scissors. A pair of haemostats was used to grip and lift the sternum, and the connective tissue and muscle underneath, including the diaphragm, were cut carefully to expose the heart. The ribs were also cut up to the clavicle, and the entire ribcage was folded back and secured. A 25-gauge blunted needle, connected to an infusion pump delivering heparinised saline (5000 U/l, Wockhardt UK Ltd., Wrexham, UK) at a rate of 30 rpm, was inserted into the left ventricle of the heart or into the ascending aorta. The needle was clamped in position using a small, curved haemostat, and a small cut was made to the right atrium with a sharp scissors. The pump pressure was increased to 120–200 rpm and the rat was perfused with heparinised saline for approximately 2 minutes or until clearance of blood from the organs was observed. The reservoir supplying the infusion pump was then switched from heparinised saline to ice-cold paraformaldehyde (Sigma-Aldrich, Dublin, Ireland), 4% in phosphate-buffered saline (pH 7.4). The rat was perfused for approximately 6 minutes, with a vigorous muscle reaction during the first minute indicating fixation. Brains and spinal cords were removed (as per Section 2.2.6.2) and post-fixed in 4% paraformaldehyde overnight. They were then transferred to a cryoprotectant (sucrose solution 25% in phosphate-buffered saline, sodium azide). Brains and spinal cords were stored in sucrose at 4°C until ready for cryosectioning. Full details on preparation of solutions are described in Appendix A.
2.2.6.2 Tissue collection

Brains and spinal cords were harvested from both perfused and non-perfused rats.

Removal of brains
Following decapitation, an incision was made along the top of the head and the skin was pulled back to expose the skull. The optic ridge between the eyes was broken with a rongeur. A shallow cut was made along the midline from the back of the skull, and the parietal and frontal bones were carefully peeled back. The optic nerve was teased out and the whole brain removed from the skull using a fine forceps.

Brain dissection
Immediately after decapitation, fresh brains (from non-perfused rats) were removed from the skull and dissected on an ice-cold plate. The regions isolated were: the frontal cortex, the hypothalamus, the cerebral cortex, the striatum, the amygdaloid cortex, the hippocampus, the periaqueductal grey (PAG), the thalamus and the cerebellum. With the exception of the hypothalamus and the PAG, all of the regions were medially divided into right and left sections. The tissue was then weighed, snap-frozen on dry ice and stored at $-80^\circ\text{C}$ for subsequent analysis.

Removal of spinal cords
The rat carcass was laid in a prone position and an incision was made down the length of the back. A toothed forceps was used to pull away the muscle until the spinal column was visible. A small, sharp rongeur was used to make two cuts, lateral to the cord, in the vertebra such that the dorsal part of the bone could be removed gently. Thoracic and lumbar vertebrae were removed, and the cord was extracted from the spinal column by carefully lifting it and cutting the attached nerve-endings. The L4–L6 region was isolated, weighed, and snap-frozen on dry ice, either whole or cut sagittally and horizontally, resulting in right and left dorsal and ventral segments. Tissues were stored at $-80^\circ\text{C}$ for subsequent processing.
2.2.7 Histology and immunohistochemistry

2.2.7.1 Isolectin B4 (IB4) staining

IB4 binds to a glycoprotein located on C-fibre nociceptors, and a qualitative decrease in staining in the dorsal horn of the spinal cord in nerve-injured animals on the side ipsilateral to ligation has been demonstrated previously (Munglani et al., 1995). To visualise IB4 expression in the spinal cord, the fixed L4–L6 region was cut into 30µm-thick transverse sections using a cryostat (Microm International, Walldorf, Germany). Sections were free-floated in 5% sucrose/0.1% sodium azide in phosphate-buffered saline (PBS, 0.1 M, pH 7.4). Sections were given three 5-minute washes in PBS and incubated overnight with FITC-conjugated Grifonia (Bandeiraea) Simplicifolia Lectin 1, IB4 (Vector Laboratories, Peterborough, UK) diluted 1:1000 in PBS. The sections were given a further three washes, mounted and coverslipped on Superfrost®-charged microscope slides (Fisher Scientific, Dublin, Ireland) using a fluorescent mounting medium, Gelmount™ or Fluoromount™ (Sigma-Aldrich, Dublin, Ireland). Sections were visualised using fluorescence microscopy (Olympus Inverted Microscope with structured light illumination), and depletion was assessed qualitatively by an experimenter blind to the treatment procedure of the rats. In Chapter 3, IB4 intensity was also quantified with the aid of ImageJ analysis software. The integrated density of a fixed area was measured in the ipsilateral and contralateral portions of the dorsal horn of the spinal cord, and the ipsilateral density was expressed as a percentage of the contralateral density.

2.2.7.2 Synaptophysin immunohistochemistry

Whole, perfused brains were removed from the cryoprotective sucrose solution, frozen and cut into 10 µm coronal sections. Sequential sections were taken from approximately bregma 3.70 so as to include the prelimbic region of the PFC, and from approximately bregma −3.14 so as to include the CA1 region of the
hippocampal formation. For PFC sections, a series of six slides was collected with six sections per slide, and for hippocampal sections, a series of nine slides with four sections per slide was collected. The sections were mounted directly onto Superfrost\textsuperscript{\textregistered}-charged microscope slides. Sections were given three 10-minute washes in PBS (0.1 M, pH 7.4) and incubated at room temperature for 2 hours with a blocking solution of 3% normal rabbit serum (Sigma-Aldrich, Dublin, Ireland) and 0.2% Triton-X (Sigma-Aldrich, Dublin, Ireland) in PBS. Sections were then incubated overnight in a humidity chamber with the primary antibody, monoclonal anti-synaptophysin (Millipore Ireland BV, Carrigtwohill, Co. Cork, Ireland), diluted 1:1000 in blocking solution. Sections were again washed three times in PBS before being incubated for 3 hours with the secondary antibody, AF488-conjugated rabbit anti-mouse IgG (Invitrogen, supplied by Bio-Sciences Ltd., Dun Laoghaire, Co. Dublin, Ireland), diluted 1:100 in blocking solution. Sections were washed twice more (2 × 10 minutes) and then coverslipped with the fluorescent mounting medium, Fluoromount (Sigma-Aldrich, Dublin, Ireland). The protocol used was adapted from those described previously (Foley et al., 2008; Yamanaka et al., 2007). Pilot experiments were carried out to determine the optimum concentrations of primary and secondary antibodies. To confirm the specificity of the antibodies used, the procedure was repeated in the absence of either the primary antibody or the secondary antibody. No specific synaptophysin staining was observed in either case. Images were obtained using an Olympus FluoView\textsuperscript{TM} 300 confocal microscope with a 60× oil-immersion objective lens. Six sections from each rat were imaged and four images were obtained for each region of interest for each hemisphere. For each image, serial z-sectioning was performed, yielding 20 optical sections, each of thickness 0.5 \( \mu \)m. The optical sections were combined through the z-axis into a compressed single z-stack image. Z-stack images were analysed with the aid of McMaster Biophotonics Facility (MBF) ImageJ software to determine the density of synaptophysin.
2.2.8 Western immunoblotting

The protocol used for western immunoblotting was similar to that described by Butler et al. (2008), with some modifications. Frozen brain tissue was lysed in 400 µl of lysis buffer (80 mM sodium β-glycerophosphate, 1 mM dithiothreitol, 1 mM sodium fluoride, pH-adjusted to 7.6) containing protease inhibitor cocktail 1 µl/1000µl of buffer (Sigma-Aldrich, Dublin, Ireland). The tissue was homogenised in a 1.5 ml microcentrifuge tube and centrifuged at 14,000 g for 15 minutes at 4°C. The supernatant was collected and the concentration of protein was determined using a Bradford protein assay (Bradford, 1976). Samples were diluted in sufficient ice-cold lysis buffer to equalise protein concentrations to 80 µg in 60 µl, and 20 µl of sample buffer (1 M tris-HCl, 1.84% sodium dodecyl sulphate (SDS), 8% glycerol, 1% bromophenol blue and 5% 2-mercaptoethanol) was then added to yield a final protein concentration of 1 µg/µl. The lysates were heated to 95°C for 5 minutes. The proteins (20 µg in 20 µl of each sample) were then separated under reducing conditions by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% polyacrylamide gels and electroblotted on to a nitrocellulose protran membrane (0.2 µm; Bio-Rad Laboratories, supplied by Alpha Technologies, Wicklow, Ireland). The membranes were transferred to a blocking solution (1% milk formula (Aptamil™), 0.1% Tween 20 in tris-buffered saline (TBS), pH 7.6) for 1 hour and then incubated overnight at 4°C with primary antibody, monoclonal anti-synaptophysin (Millipore Ireland BV, Carrigtwohill, Co. Cork, Ireland), diluted 1:1000 in 1% milk formula, 0.1% Tween 20 in TBS. Membranes were washed three times (3 × 5 minutes) in washing solution (0.1% Tween 20 in TBS) and then agitated at room temperature in secondary antibody solution, anti-mouse IgG (Fab specific)-peroxidase goat antibody (Sigma-Aldrich, Dublin, Ireland). Two 5-minute washes in washing solution and a final wash in 1X TBS (200 mM Trizma®-base, 1.37 M NaCl in distilled water) were performed. Chemiluminescence and image acquisition were performed under safe-light conditions. Membranes were exposed to chemiluminescent reagents (SuperSignal®
West Dura Extended Duration Substrate 34075, Thermo Fisher Scientific Inc., Rockford, IL, USA) for 2–5 min, followed by exposure to the G:BOX iCHEMI image analyser (Syngene, Cambridge, UK) for 3–15 min. Membranes were then stripped (25 mM glycine-HCl pH 2, 20% SDS) for 15 minutes to remove bound antibody. Three washing steps (3 × 5 minutes) were performed in washing solution (0.1% Tween 20 in TBS), and the blocking and antibody incubation steps were repeated except that the primary antibody was monoclonal mouse anti-β-actin antibody diluted 1:10 000 (Sigma-Aldrich, Dublin, Ireland). The concentrations of primary antibodies used were based on manufacturer’s guidelines and on published literature measuring synaptophysin by western blot in rodent brain lysates (Bie et al., 2009; Dumont et al., 2011, 2009; Wang et al., 2011). Preliminary experiments were also conducted to determine the optimal concentration of anti-synaptophysin. Protein bands were quantified using densitometry with the aid of ImageJ software. Background integrated density values were computed and subtracted from band values to calculate corrected integrated density values. The corrected values for synaptophysin were then normalised with respect to β-actin. Normalised values for each sample were then divided by those for the control group and results were expressed as percentage of control. Images were inverted for presentation using an inverted lookup table.

See Appendix A for preparation of solutions.

2.2.9 High-performance liquid chromatography with electrochemical detection for measurement of brain monoamines

Quantification of monoamines by HPLC was carried out according to methods described previously (Ford et al., 2008; Roche et al., 2007). Gross-dissected, weighed brain regions were placed on ice and sonicated in 1 ml of mobile phase buffer (0.1 M citric acid, 0.1 M sodium dihydrogen orthophosphate, 1.4 mM 1-octanesulphonic acid, 0.01 mM ethylenediaminetetraacetic acid and 10% methanol v/v, pH-adjusted to 3.5) spiked with 2 ng/20 µl of the internal standard, N-methyl-
5-hydroxytryptamine (N-methyl-5HT). The tissue homogenates were centrifuged at 14,000 g for 15 minutes at 4°C, and a 20 µl sample of supernatant was injected onto a reverse-phase analytical column (Licrosorb C18, length 250 cm and internal diameter 4.0 mm, Phenomenex, Macclesfield, UK). The eluate was analysed using electrochemical detection (Shimadzu LECDC6A) coupled with a Merck-Hitachi® D-2000 integrator. A typical chromatogram produced by the system is shown in Figure 2.9. Chromatograms were analysed with Agilent ChemStation for LC and LC/MS Systems (Agilent Technologies, Cork, Ireland).

![Chromatogram showing peaks and retention times corresponding to the elution of monoamines.](image)

**Figure 2.9**: Representative chromatogram showing peaks and retention times corresponding to the elution of monoamines.

The components of interest (5-hydroxytryptamine (5-HT, serotonin), 5-hydroxyindole-3-acetic acid (5-HIAA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and noradrenaline (NA)) were identified from the relative elution times at which the peaks occur in the chromatogram. Standard mixtures, containing 2 ng/20 µl of each monoamine or metabolite to be analysed and 2 ng/20 µl of N-methyl-5HT (internal standard), were run through the system daily, before, during (typically every 10 samples) and after injection of tissue samples. The chromatogram shown in Figure 2.9 was actually obtained from one such standard run; the peaks have different heights even though the concentrations are all equal,
and the standard runs were therefore used to determine a relative retention factor for each component to take account of this. The relative retention factor, $f_R$, is defined as

$$f_R = \frac{c_{IS}/h_{IS}}{c_M/h_M}$$

where $c_{IS}$ and $c_M$ are the concentrations of internal standard (N-methyl-5HT) and monoamine/metabolite, respectively, in the standard mixture, and $h_{IS}$ and $h_M$ are the heights of the corresponding peaks in the chromatogram.

For a tissue sample run with unknown concentrations of monoamines/metabolites and a known concentration $c_{IS}'$ of the internal standard, the concentrations $c_M'$ of the monoamines/metabolites can be then calculated from

$$c_M' = \left( \frac{h_M'}{f_R} \right) \frac{c_{IS}'}{h_{IS}'}$$

where $h_{IS}'$ and $h_M'$ are the peak heights for the internal standard and monoamine, respectively, in the sample run chromatogram. Using the known weight of tissue contained in 1 ml of the homogenate, the results were then expressed in ng/g of fresh tissue.

### 2.2.10 Human participants

Experimental procedures involving human participants were carried out with the approval of the Research Ethics Committee of the National University of Ireland Galway and the Galway University Hospitals Research Ethics Committee. Patients with a diagnosis of chronic (minimum of 3 months) neuropathic pain or radiculopathy were selected from the database of patients attending the Pain Clinic at University Hospital Galway, with the assistance of clinic staff. Recruitment of control participants was achieved through placement of advertisements in public places and in the local and national media (see Appendix E, Figure E.1). The suitability criteria for participants are outlined in Table 2.3 and discussed in detail in Chapter 7. Before any assessments were carried out, participants were provided with an information sheet (Appendix E, Sections E.5–E.6), and the pro-
procedures were explained in detail by the experimenter. Participants were advised that they could withdraw at any point, without giving a reason, and that a decision to do so would not affect their rights or the care they received. Any questions arising were answered by the experimenter, and participants were then asked to give written consent (page 457, Appendix E). In addition, all participants were informed that the data obtained would be confidential, anonymised and stored securely.

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chronic Pain Patient Group</strong></td>
<td>• Diagnosis of chronic (minimum of 3 months) neuropathic pain or radiculopathy</td>
</tr>
<tr>
<td></td>
<td>• Aged 18 years or over</td>
</tr>
<tr>
<td></td>
<td>• Sufficient English language ability to complete the tests</td>
</tr>
<tr>
<td></td>
<td>• Traumatic brain injury, psychiatric illness, substance abuse, epilepsy or seizures, or diabetes</td>
</tr>
<tr>
<td></td>
<td>• Pre-existing cognitive impairment (such as a learning disability, a brain injury or dementia)</td>
</tr>
<tr>
<td><strong>Control Group</strong></td>
<td>• Aged 18 years or over</td>
</tr>
<tr>
<td></td>
<td>• Sufficient English language ability to complete the tests</td>
</tr>
<tr>
<td></td>
<td>• As above, with the addition of a diagnosis or history of chronic pain</td>
</tr>
</tbody>
</table>

Table 2.3: Criteria for selection of participants.

To ensure that all procedures in the clinical study were conducted to an appropriate professional standard, the author undertook classes in psychometric evaluation and supervised practice in test administration, and all participant responses were checked independently in accordance with the guidelines of the Research Ethics Committees.
2.2.11 Participant demographics

Demographic information, including age, gender and number of years of education, was collected for all participants using a standard form (page 459, Appendix E). In addition, participants, where applicable, were asked to estimate the length of time since they had last consumed nicotine, caffeine and/or alcohol.

2.2.12 Pain measures

Participants in the patient group were asked to complete the Chronic Pain Grade (CPG) Questionnaire (page 462, Appendix E), which is used to derive scores for pain intensity, pain-related disability and an overall chronic pain grade classification (Von Korff et al., 1992). Item 1 of the questionnaire also provides a measure of present pain intensity. The CPG has been widely used in chronic pain research (Dunn et al., 2008; Elliott et al., 1999; Raftery et al., 2011). In addition, pain patient participants were asked to indicate painful areas on a manikin diagram (Figure 2.10) and to estimate their pain chronicity (i.e., the number of months since the diagnosis of their pain). Patients’ analgesic medication and any adjunctive pain management techniques were also recorded.

2.2.13 Depression and anxiety measures

2.2.13.1 Patient health questionnaire

We used the 9-item Patient Health Questionnaire (PHQ-9) depression scale (©Pfizer Inc. 1999). The PHQ-9 (page 464, Appendix E) is widely used as a measure of depressive symptoms (Kroenke et al., 2001; Kroenke and Spitzer, 2002). Scores were computed to give a tentative diagnosis of depression and a measure of symptom severity. The questionnaire responses also gave an indication of symptom-related functional impairment.
2.2.13.2 Spielberger State Anxiety Inventory

The level of anxiety at the time of cognitive testing was quantified using the “state” portion of the State-Trait Anxiety Inventory (STAI), Form Y (Spielberger, 1983), reproduced on page 465 (Appendix E). This provides a measure of “state” or current anxiety at the time of completing the questionnaire.

2.2.14 General intellect

Participants’ intelligence quotient (IQ) was estimated using the Digit-Symbol Coding and Information subtests of the Wechsler Adult Intelligence Scale-III (WAIS®-III, Wechsler (1997b)), the most widely used test of adult intelligence. Estimated full-scale IQ obtained using this dyadic short form of the WAIS-III has been shown previously to correlate with IQ values obtained using the full 12-subtest scale (Ringe et al., 2002). The Digit-Symbol Coding subtest consisted
of nine digit-symbol pairs followed by a list of digits. Participants were required to fill in the symbol corresponding to each digit as quickly as possible on a standard form (page 466, Appendix E). For the Information subtest, the examinee was required to answer a series of questions about factual information relating to general knowledge about common events, objects, places and people (page 467). Raw scores for both subtests were converted to age-adjusted scaled score equivalents using the WAIS-III Administration and Scoring Manual (Wechsler, 1997a). The classifications of the scaled scores are: 8–12, average range (25th–75th percentile); 10, absolute average (50th percentile); 7, below average (16th percentile); 6, borderline impairment (9th percentile); 5 and below, impaired (5th percentile). Full-scale IQ was then estimated from the sum of the scaled scores using the extrapolation tables taken from Sattler and Ryan (2001).

2.2.15 Cognitive measures

2.2.15.1 Verbal memory

The Logical Memory subtests I and II of the Wechsler Memory Scale-III (WMS III, Wechsler (1997c)) were used to assess short- and long-term verbal memory and recognition memory. Two different stories (A and B) were read to the subject and immediately afterwards the subject was asked to retell the story from memory. After an interval of approximately 25 minutes, the subject was again asked to recall as many details as possible from both stories A and B. For recognition, the subject is required to give “Yes” or “No” answers to a set of 30 questions relating to stories A and B. The stories and the associated questions are included in Appendix E (pages 472–474). Participants were scored on the accuracy of the story recall and number of correct responses to recognition questions. Raw scores were converted to age-adjusted scaled score equivalents using the WMS-III Administration and Scoring Manual (Wechsler, 1997d).
2.2.15.2 Spatial memory

The spatial span subtest of the WMS-III was used to measure short-term spatial memory capacity. The test was administered using the spatial span board, which consists of 10 cubes with the numbers 1 to 10 printed on the sides of the cubes facing the examiner (see Figure 2.11). For spatial span forward, the examiner tapped a specified sequence pattern and then asked the participant to tap the same sequence. For spatial span backward (reverse), the participant was asked to tap the sequence in reverse order. The sequence length increased until the participant could no longer replicate the sequence correctly. Raw scores were converted to age-adjusted scaled score equivalents using the WMS-III Administration and Scoring Manual (Wechsler, 1997d).

![Spatial span board: (a) Rear view, as seen by tester; (b) Front view, as seen by test subject.](image)

2.2.15.3 Attention/vigilance

The Continuous Performance Test – Identical Pairs (CPT-IP), adapted from the MATRICS (Measurement and Treatment Research to Improve Cognition in Schizophrenia) Consensus Cognitive Battery (MATRICS Assessment Inc., Los Angeles, CA, USA) (Nuechterlein and Green, 2004), was used to measure attention and vigilance. This computerised test required the participant to monitor a series of 4-digit numbers as they appeared briefly on the computer screen. The participant responded to the sequential appearance of identical pairs of numbers by clicking a mouse as quickly as possible. The number of correct responses, the
number of incorrect responses and the reaction time were recorded automatically. Incorrect responses were categorised as “false alarms” (i.e., responses to similar, but not identical, numbers presented in sequence) or random responses.

2.2.15.4 Executive function

Executive function was assessed using the Wisconsin Card Sorting Test® – Computerised Version 4 (WCST-CV4, PAR Inc., Lutz, FL, USA). This test measures the subjects’ ability to adapt to changing schedules of reinforcement (i.e., the “rules” about the task) and thus assesses cognitive flexibility, a key component of executive functioning (Berg, 1948). The computerised version used in this study presents the participant with four key cards and a stimulus card (see Figure 2.12). The cards were matched according to one of three categories: colour, shape or quantity of items on the card. Matching is achieved by placing the cursor on the key card selected and left clicking the mouse. The participants were not told how

![Figure 2.12: Screenshot of the user interface for the Wisconsin Card Sorting Test® – Computerised Version 4 (PAR Inc., Lutz, FL, USA).](image-url)
to match the cards but they were given verbal and visual feedback on whether each match was “right” or “wrong”. The category by which the cards were to be matched changed without warning after presentation of every ten stimulus cards; the subject was required to recognise the changed “rules” and identify the new presentation pattern.

2.2.15.5 Perceived impact of pain

Patients’ subjective ratings of the perceived impact of pain on cognitive function (concentration, memory, problem solving and decision making) were also recorded.

2.2.16 Statistical analyses

Full details of the specific statistical analyses used are provided in the Materials and Methods section of each results chapter (Chapters 3 – 7). Note that as both non-parametric and parametric statistics are used as appropriate throughout the thesis, the majority of behavioural data have been presented as means ± standard error of the mean (SEM), for consistency and clarity, and to allow comparison of figures within and between chapters. For behavioural tests, such as those measuring sensory hypersensitivity and the water-maze tasks, a repeated-measures design was employed. As a consequence, there are cases in which the data set as a whole is non-parametric, largely due to a particular time point or experimental subject, while the rest of the data follows a normal distribution. Therefore, the mean ± SEM is still an appropriate representation of the data.
Chapter 3  Characterisation of Cognitive Behaviour in the SNL Model of Neuropathic Pain in Young Rats

3.1 Introduction

As discussed at length in Chapter 1, the weight of evidence from both the clinical and preclinical literature suggests that cognition is affected in chronic pain conditions. Chronic pain patients subjectively report difficulty with memory and concentration, and objective measures of cognitive ability show that patients are impaired when compared with healthy controls. Approximately one in six cases of chronic pain is predominantly neuropathic in origin (Torrance et al., 2006). Neuropathic pain is generally characterised by somatosensory sensitisation including allodynia (pain in response to a stimulus that does not normally provoke pain) and hyperalgesia (increased response to a stimulus which is normally painful). As well as its sensory symptoms, neuropathic pain has been shown to be associated with effects on cognitive functioning. One of the largest clinical investigations ($n = 1519$) into the effect of pain on cognition focused on neuropathic pain and found that patients with neuropathic pain performed poorly compared with the reference sample on a measure of broad cognitive functioning, and significantly worse than those with mixed neuropathic and nociceptive pain (Povedano et al., 2007). Other studies have also shown cognitive deficits in neuropathic pain patients in a variety of cognitive domains (Grisart and Van der Linden, 2001; Karp et al., 2006; Oosterman et al., 2011; Ryan et al., 1993; Tassain et al., 2003; Veldhuijzen et al., 2006b). Despite a number of hypotheses, as outlined in Chapter 1, the precise mechanisms by which pain affects cognitive functioning remain poorly understood. As such, an animal model of this clinical phenomenon would be a use-
ful tool for the investigation of the neural substrates involved. Prior to 2008, the majority of preclinical studies that attempted to model cognitive deficits associated with pain were conducted in inflammatory pain models (Boyette-Davis et al., 2008; Cain et al., 1997; Lindner et al., 1999; Millecamps et al., 2004). Adjuvant-induced inflammatory pain was found to be associated with deficits in an operant delayed non-matching to position task (Cain et al., 1997; Lindner et al., 1999). Chronic visceral pain was shown to impair novel object recognition (Millecamps et al., 2004), and deficits in attention were observed in the formalin model of persistent inflammatory pain (Boyette-Davis et al., 2008).

Previous work in our laboratory utilised the rat streptozotocin model of diabetes, and resulting hyperalgesia of the paws (mimicking the symptoms of diabetic neuropathy), to investigate the effect of neuropathic pain on cognition. Streptozotocin treatment was associated with increased mechanical sensitivity of the hindpaws and impaired spatial learning in the Morris water maze (Moriarty et al., 2009). However, interpretation of these findings is confounded because of the complex set of physiological changes associated with induction of the diabetic model. For example, hyperglycaemia may cause neurovascular changes which could impact negatively on cognition. Therefore, it is difficult to draw conclusions regarding the direct relationship between neuropathic pain and cognition.

A number of surgical models of neuropathic pain based on damage to the sciatic nerve have been developed and are associated with fewer pathophysiological features than the streptozotocin-induced diabetes model. These include the chronic constriction injury model (Bennett and Xie, 1988), the partial sciatic nerve ligation model (Seltzer et al., 1990), the spinal nerve ligation model (Kim and Chung, 1992) and the spared nerve injury model (Decosterd and Woolf, 2000). In a comparison of three of these models by (Kim et al., 1997), all were associated with behavioural signs of neuropathic pain over a similar time course, with spinal nerve ligation (SNL) showing the most robust expression of mechanical allodynia. Furthermore, this model is not generally accompanied by significant deficits in motor
CHAPTER 3. SNL MODEL IN YOUNG RATS: COGNITIVE BEHAVIOUR

activity (Kim and Chung, 1992; Chung et al., 2004). This is a desirable characteristic of a pain model used to measure cognition, as many commonly used behavioural tests of cognition involve a motor component. Based on these findings, the SNL model was chosen to model a neuropathic pain-like condition in this, and all subsequent experiments.

Cognition broadly refers to learning and memory processes, and animal models of cognitive behaviour “analyse the capability of an animal to form representations of the outer world that lead to altered behavioural performances on subsequent confrontation with distinct stimuli and situations” (Sousa et al., 2006). Different cognitive tests are used to investigate processes relying on distinct brain regions. The measures used to assess cognition in this experiment were novel object recognition, T-maze spontaneous alternation and Morris water maze tasks, and were chosen with relevance to brain regions involved. In Chapter 1, a theoretical model of how pain-related cognitive impairments may occur was proposed. Pain uses neuroanatomical resources, alters neural plasticity, and affects expression and activity of a variety of chemical and cellular neuromediators. Such alterations occurring in cognitive-associated brain regions may result in cognitive impairment. Brain regions common to the processing of pain and cognitive information include the PFC and the hippocampus. The hippocampus is required for the integration and processing of sensory features of an event, and so is important in spatial learning tasks such as the T-maze and the Morris water maze in rodents. The hippocampus may also be involved in contextual and spatial aspects of object recognition (Mumby et al., 2002; Wood et al., 1993). The PFC is particularly relevant in behavioural tasks that require intact working memory (Dalley et al., 2004). Examples include delayed alternation, such as that assessed in the T-maze, and delayed non-matching to sample tasks, a component of object recognition.

The specific aims of this study were:

- To characterise the neuropathic pain-like behaviours associated with the
SNL model, in our hands, over a period of 2 weeks, as the model had not been reproduced previously in our laboratory.

- To characterise the behavioural cognitive profile of rats that had undergone SNL surgery and to compare their performance to that of sham-operated controls in a battery of behavioural cognitive tests.

- To verify the successful ligation of the L5 and L6 spinal nerves using post-mortem IB4 staining of transverse sections of the spinal cord.

3.2 Methods

3.2.1 Animals

For this experiment, twenty-one young adult male Sprague-Dawley rats were used. Rats were 4–7 weeks old on arrival at our facility and weighed 150–180 g. As described in Chapter 2, experimental procedures were conducted with the approval of the appropriate agencies and according to relevant ethical guidelines. Animals were housed under standard condition of light and temperature and food and water were available ad libitum.

3.2.2 Experimental protocol

Rats were habituated to the facility for a period of 4–8 days prior to surgery. They were randomly assigned to either SNL ($n = 12$) or sham ($n = 9$) treatment groups. Surgeries were carried out as described in Chapter 2. Both groups were tested for the development of mechanical allodynia using the von Frey method (as described in Chapter 2) over a period of 2 weeks, on days 1, 9, 11 and 13 post surgery. The arena used in this experiment consisted of a six-chambered Perspex arena with dimensions 16 cm ($l$) × 20 cm ($w$) × 43 cm ($h$). No pre-test day habituation period or baseline measures of mechanical sensitivity were carried out. The Hargreaves test, also described in Chapter 2, was used to determine whether
thermal hyperalgesia was expressed on day 10 post surgery. Similar to von Frey testing, no pre-test day habituation or baseline measurements were performed in this study. Cognitive performance of SNL and sham rats was then characterised using three different behavioural tests: the novel object recognition test, the T-maze spontaneous alternation test and the Morris water maze test. As described in Chapter 2, novel object recognition measures recognition memory and involves a 2-day protocol of habituation and testing. Habituation was carried out on day 14 post SNL or sham surgery, and testing on day 15 post surgery. One rat was excluded from the novel object recognition analyses, because it failed to explore both objects in the familiarisation period (Exposure 1) and therefore would not have been able to distinguish between novel and familiar objects. The T-maze test of spontaneous alternation, assessing spatial memory, was carried out on days 22 and 23 post surgery. Morris water maze testing in this experiment consisted of a cued test performed on day 26, 28 or 30 post surgery, a 5-day acquisition training phase carried out between days 28 and 36 post surgery, and a probe trial which was carried out on day 33, 35 or 37 post surgery. Testing was carried out in the smaller of the two mazes described in Chapter 2. At the end of the experimental period (between days 40 and 44 post surgery), rats were sacrificed by transcardial perfusion with heparinised saline followed by 4% paraformaldehyde in phosphate buffer as described in Chapter 2. Brains and spinal cords were removed and stored for subsequent processing. The study timeline is illustrated in Figure 3.1. Levels of IB4 in the dorsal horn of the spinal cord were assessed qualitatively as described in Chapter 2. This was considered a non-behavioural verification of successful ligation of the L5 and L6 spinal nerves.

3.2.3 Statistical analysis

Non-continuous data (i.e., the % withdrawal response to individual filaments in von Frey testing) were analysed non-parametrically by Friedman’s analysis of variance (ANOVA) by ranks followed by Mann-Whitney U tests or Wilcoxon
Figure 3.1: Experimental timeline.
signed-ranks tests. All of the other behavioural data was tested for normality and homogeneity of variance, using Shapiro-Wilk and Levene tests, respectively. If data were not normally distributed, root-transformation or transformation by negative inversion were used where possible. If data could not be transformed, they were analysed similarly to non-continuous data. Parametric data were analysed by repeated-measures ANOVA, two-way ANOVA or unpaired two-tailed $t$-test. Fisher’s least-significant-difference (LSD) tests were used to make post hoc comparisons where appropriate. Pearson correlations were used to investigate the relationship between IB4 intensity and von Frey response thresholds. The level of statistical significance was set at $p = 0.05$. Data were analysed using SPSS software for Windows and results were depicted graphically with the aid of GraphPad Prism® software. For clarity of presentation, data are expressed as means ± SEM.

3.3 Results

3.3.1 Sensory testing

3.3.1.1 Mechanical alldynia: von Frey testing

Mechanical alldynia is a characteristic feature of neuropathic pain and its development was assessed post surgery using the von Frey test method as described in Chapter 2. We measured both the threshold to mechanical stimulation and the % response to individual von Frey filaments (the responses to the 6 g filament are presented below as an example) as indices of sensitivity to mechanical stimuli in ipsilateral and contralateral hindpaws. Firstly, we compared the threshold responses in the ipsilateral paw of SNL and sham control rats. Repeated-measures ANOVA revealed a significant effect of time ($F_{(3,57)} = 13.821, p < 0.001$), surgical treatment ($F_{(1,19)} = 8.634, p = 0.002$) and a time × surgical treatment interaction ($F_{(3,57)} = 4.834, p = 0.005$). SNL surgery was associated with a decrease in the filament weight (i.e., the mechanical force) required to produce a 50% response, in the hindpaw ipsilateral to ligation, on day 1 ($p < 0.001$) and on day
Figure 3.2: Effect of SNL surgery on mechanical response threshold in ipsilateral paws. SNL rats expressed mechanical allodynia compared with sham controls on days 1 and 11 post surgery. Threshold was decreased in the sham group on days 9, 11 and 13 compared with day 1. Data are expressed as means ± SEM, n = 9 – 12. Fisher’s LSD post hoc analysis: ***p < 0.001, *p < 0.05 SNL vs. sham control; **p < 0.01 sham days 9, 11 and 13 vs. sham day 1.

11 (p < 0.05) post surgery, compared with the sham control group (Figure 3.2). These data suggest expression of mechanical allodynia in the ipsilateral paw of SNL rats compared with sham controls at these time points. Compared with the first test session, there was a significant decrease in the threshold in the sham group during all subsequent test sessions (p ≤ 0.001). This temporal decrease in sham response threshold is a feature observed throughout testing, and is discussed in Section 3.4.

The % response to the 6 g filament was analysed by Friedman’s ANOVA by ranks which revealed a significant overall effect ($\chi^2(7) = 16.612$, $p = 0.02$). There were significant increases in the % withdrawal response of the SNL group to the subnoxious 6 g filament on days 1, 11 and 13 (p ≤ 0.05) compared with sham-operated rats, again suggesting expression of mechanical alldynia in the ipsilateral paw of SNL rats. There was also a significant increase in the % response in the sham-operated group on day 9 post surgery compared with day 1 post surgery ($p < 0.01$; Figure 3.3). Possible reasons for this increase are discussed in Section 3.4.
CHAPTER 3. SNL MODEL IN YOUNG RATS: COGNITIVE BEHAVIOUR

Figure 3.3: Effect of SNL surgery on the % response to mechanical stimulation (6 g filament) of the ipsilateral hindpaw. SNL rats expressed mechanical allodynia compared with sham controls on days 1, 11 and 13 post surgery. % response increased in the sham group on day 9 compared with day 1. Data are presented as means ± SEM, $n = 9 - 12$. Mann-Whitney U tests: *$p \leq 0.05$ SNL vs. sham; Wilcoxon signed-ranks test: ##$p < 0.01$ sham day 9 vs. sham day 1.

Figure 3.4: Mechanical response threshold in the ipsilateral and contralateral paws following SNL surgery. SNL rats expressed mechanical allodynia in the ipsilateral paw compared with the contralateral paw at all post-surgery time points. Threshold decreased in the contralateral paw on days 9, 11 and 13 compared with day 1. Data are expressed as means ± SEM, $n = 9 - 12$. Fisher’s LSD post hoc analysis of negatively inverted data: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ SNL ipsilateral vs. SNL contralateral, ###$p < 0.001$ SNL contralateral days 9, 11 and 13 vs. SNL contralateral day 1.
Figure 3.5: Effect of SNL surgery on mechanical response threshold in the contralateral paws. Data are expressed as means ± SEM, n = 9 – 12. Threshold was decreased in the contralateral paw of both sham and SNL rats on days 9, 11 and 13 compared with day 1. Fisher’s LSD post hoc analysis of negatively inverted data: ###$p < 0.001$ sham days 9, 11 and 13 vs. sham day 1, δδδ$p < 0.001$ SNL days 9, 11 and 13 vs. SNL day 1.

Figure 3.6: Mechanical response threshold in ipsilateral and contralateral paws following sham surgery. On day 9 post surgery, threshold was lower in the ipsilateral paw than in the contralateral paw. Threshold was decreased in the ipsilateral and contralateral paws of sham rats on days 9, 11 and 13 compared with day 1. Data are expressed as means ± SEM, n = 9 – 12. Fisher’s LSD post hoc analysis: *$p < 0.05$ ipsilateral day 9 vs. contralateral day 9, ###$p < 0.01$ contralateral days 9, 11 and 13 vs. contralateral day 1, δδδδ$p < 0.001$ ipsilateral days 9, 11 and 13 vs. ipsilateral day 1.
We also compared the 50% withdrawal threshold in the ipsilateral and contralateral paws in the SNL group. Data were transformed by negative inversion. Repeated-measures ANOVA revealed main effects of time ($F_{(3,66)} = 12.628, \ p < 0.001$), surgical treatment ($F_{(1,22)} = 68.038, \ p < 0.001$) and a time × surgical treatment interaction ($F_{(3,66)} = 3.444, \ p = 0.022$). The response threshold was decreased in the ipsilateral paw of SNL-operated rats compared with the contralateral paw at all of the time points investigated ($p < 0.01$). There was a decrease in the contralateral response threshold at all time points compared with the first test session ($p < 0.001$). The hypersensitivity of the ipsilateral paw compared with the contralateral paw suggests that the SNL surgery unilaterally induced neuropathic pain-like behaviours (Figure 3.4).

To investigate possible mirrored neuropathic pain-like responses in the contralateral paw, we compared the threshold response in the contralateral paw of SNL and sham control rats (Figure 3.5). Data were transformed by negative inversion and a repeated-measures ANOVA revealed a significant effect of time ($F_{(3,57)} = 36.742, \ p < 0.001$). There was no significant effect of surgical treatment and no time × surgical treatment interaction effect. There was a decrease in the threshold over time in both SNL and sham groups, with lower values on days 9, 11 and 13 compared with day 1 post surgery ($p < 0.001$). These results suggest that there were no differences in the sensitivity of the contralateral hindpaws of SNL and sham rats to mechanical stimulation. Finally, we compared the threshold response in ipsilateral and contralateral paws in the sham control group, to determine within-subject responses to sham surgery. Repeated-measures ANOVA revealed a significant effect of time ($F_{(3,42)} = 31.087, \ p < 0.001$). The threshold response was significantly lower in the ipsilateral paw of sham controls than in the contralateral paw on day 9 post surgery ($p < 0.05$). Possible reasons for this difference are discussed in Section 3.4. A decrease in the response threshold was also observed on post-surgical days 9, 11 and 13 compared with the day 1 post-surgery test session ($p < 0.001$) in both ipsilateral and contralateral paws (Figure 3.6).
3.3.1.2 Thermal hypersensitivity: Hargreaves testing

Thermal hypersensitivity, another hallmark of neuropathic pain, was assessed in the SNL model using the Hargreaves method (see Chapter 2) at one postsurgical time point (day 10). The latency to withdraw the paw following application of a noxious thermal stimulus was measured in both the ipsilateral and contralateral hindpaws. An unpaired two-tailed $t$-test on root-transformed data showed that SNL surgery was associated with a decrease in paw withdrawal latency in the ipsilateral paw compared with the ipsilateral paw of sham controls ($t(1,19) = 2.179$, $p < 0.05$) (Figure 3.7). Furthermore, there was a decrease in the withdrawal latency in the ipsilateral paw compared with the contralateral paw in the SNL group ($t(1,22) = 3.406$, $p < 0.01$) (Figure 3.8). These results suggest expression of thermal hyperalgesia, specifically in the ipsilateral hindpaw of SNL rats at this time point. There were no between-group differences in paw withdrawal latencies in the contralateral paw (Figure 3.9), and there was no difference between the ipsilateral and contralateral paws in the sham control group (Figure 3.10).

![Figure 3.7: Effect of SNL surgery on ipsilateral hindpaw withdrawal latency in the Hargreaves test. Paw withdrawal latency was decreased in the ipsilateral paw of SNL rats compared with sham rats. Data are expressed as means ± SEM, $n = 9 – 12$. Unpaired two-tailed $t$-test of root-transformed data: *$p < 0.05$ SNL ipsilateral vs. sham ipsilateral.](image-url)
Figure 3.8: Effect of SNL surgery on paw withdrawal latency in the Hargreaves test in SNL rats in ipsilateral and contralateral hindpaws. Paw withdrawal latency was decreased in the ipsilateral paw compared with the contralateral paw in SNL rats. Data are expressed as means ± SEM, $n = 9 - 12$. Unpaired two-tailed $t$-test of root-transformed data: **$p < 0.01$ SNL ipsilateral vs. SNL contralateral.

Figure 3.9: Paw withdrawal latency in the Hargreaves test in contralateral paws of sham and SNL rats. Data are expressed as means ± SEM, $n = 9 - 12$.

Figure 3.10: Paw withdrawal latency in the Hargreaves test in ipsilateral and contralateral paws of sham control rats. Data are expressed as means ± SEM, $n = 9 - 12$. 
3.3.2 Cognitive testing

3.3.2.1 Novel object recognition

The novel object recognition task was used to assess recognition memory in sham and SNL rats. Discrimination ratios for the identical objects in Exposure 1, and familiar and novel objects in Exposure 2 were calculated according to the formula shown in Section 2.2.4.1. Each exposure was analysed by a one-way ANOVA, followed by post hoc tests. For completeness, the total duration (in seconds) of object exploration is also presented (Figure 3.12). These data were analysed by two-way ANOVA with object and group as factors. There were no differences in object exploration in either group or between groups in Exposure 1 (Figure 3.11). In Exposure 2, both the sham and the SNL groups displayed a preference for the novel object as shown by greater discrimination ratios for the novel object than for the familiar object ($p < 0.001$ and $p < 0.05$, for sham and SNL groups, respectively). This increased preference for the novel object tended to be greater in the sham group than in the SNL group, suggesting a slight deficit in recognition memory in the SNL group compared with sham controls. However, this difference just failed to reach the level of statistical significance ($p = 0.073$) (Figure 3.11). Neither sham nor SNL rats displayed a preference for object position, i.e., left or right (discrimination ratios for Exposure 2: sham right 0.45 ± 0.1 vs. sham left 0.54 ± 0.1, difference not significant; SNL right 0.50 ± 0.07 vs. SNL left 0.50 ± 0.07, difference not significant). For duration of object exploration, two-way ANOVA revealed a main effect of object in Exposure 2 ($F_{(1,36)} = 14.22$, $p = 0.001$). Both sham and SNL rats spent longer exploring the novel object than the familiar one (*$p < 0.05$, **$p < 0.01$, novel object vs. familiar object, Fisher’s LSD post hoc analysis) (Figure 3.12). The effects of nerve ligation surgery on general behaviours and locomotor activity were examined in the 3-minute habituation period prior to object familiarisation. There were no differences between sham and SNL groups in sniffing, rearing or grooming behaviours (measured both by the frequency and total duration), or in the total exploratory behaviour defined as duration of sniffing.
Figure 3.11: Novel object recognition in sham and SNL rats. There were no differences in object discrimination in Exposure 1. In Exposure 2, sham and SNL rats expressed a preference for the novel object. The increased exploration of the novel object tended to be attenuated by SNL surgery, with SNL-operated rats exploring the novel object less than the sham controls did ($p = 0.073$). Data are expressed as means ± SEM, $n = 9–11$. Fisher’s LSD post hoc analysis: *$p < 0.05$, ***$p < 0.001$, novel object vs. familiar object.

plus duration of rearing (Figures 3.13 and 3.14). Importantly, SNL surgery did not affect locomotor activity as there was no change in the total distance moved during the habituation period compared with sham controls (Figure 3.15). This implies that differences in object exploration are likely due to altered cognitive processing rather than surgery-related impairment of movement.
Figure 3.12: Total duration of object exploration in novel object recognition. There were no differences in exploration between objects or groups in Exposure 1. In Exposure 2, both sham and SNL rats spent significantly more time exploring the novel object than the familiar one (\( **p < 0.01 \), \( *p < 0.05 \), novel object vs. familiar object, Fisher’s LSD post hoc test); there were no between-group differences. Data are presented as means ± SEM, \( n = 9 – 11 \).

Figure 3.13: Duration of general and exploratory behaviours in SNL and sham rats during novel object habituation phase. Data are expressed as means ± SEM, \( n = 9 – 11 \).
Figure 3.14: Frequency of general and exploratory behaviours in SNL and sham rats during novel object habituation phase. Data are expressed as means ± SEM, \( n = 9 - 11 \).

Figure 3.15: Locomotor activity of SNL and sham rats during novel object habituation phase. Data are expressed as means ± SEM, \( n = 9 - 11 \).
3.3.2.2 T-maze

Spontaneous alternation in a T-shaped arena was used to assess spatial memory. There were no differences in the % alternation (Figure 3.16), the % error (incorrect arm choices) (Figure 3.17) or the % omission (failure to choose an arm) (Figure 3.18) between SNL rats and sham controls. Furthermore, there were no between-group differences in the average latency to enter an arm (Figure 3.19) or the total number of arm entries (Figure 3.20). The average velocity over trials was also similar between SNL and sham groups (Figure 3.21).

Figure 3.16: % spontaneous alternation in the T-maze in SNL and sham rats. Data are expressed as means ± SEM, n = 9 – 11.

Figure 3.17: % errors in the T-maze alternation in SNL and sham rats. Data are expressed as means ± SEM, n = 9 – 11.
Figure 3.18: % trials omitted in the T-maze in SNL and sham rats. Data are expressed as means ± SEM, $n = 9 - 11$.

Figure 3.19: Average latency to arm choice in the T-maze alternation task in SNL and sham rats. Data are expressed as means ± SEM, $n = 9 - 11$.

Figure 3.20: Total number of arm entries in the T-maze alternation task in SNL and sham rats. Data are expressed as means ± SEM, $n = 9 - 11$. 
3.3.2.3 Morris water maze

The Morris water maze was used to measure spatial learning and memory. Behaviour was analysed using three distinct protocols, the cued trial, acquisition training and the probe trial. In the cued trial, the rats’ visual and swimming ability, and their recognition of the platform as an escape route, were assessed by measuring their swim speed and the latency and the path length to the visible platform. Latency to the platform was analysed by Friedman’s ANOVA by ranks which revealed an overall significant effect ($\chi^2(7) = 35.817$, $p < 0.001$), and significant effects of time in both sham ($\chi^2(3) = 17.292$, $p = 0.001$) and SNL groups ($\chi^2(3) = 18.7$, $p < 0.001$). There was a decrease in the latency to get onto the platform over the 4-trial test period in both groups, with significantly shorter latencies in trials 2, 3 and 4 compared with trial 1 ($p < 0.01$) (Figure 3.22). The path length was also analysed non-parametrically with Friedman’s ANOVA by ranks revealing a significant overall effect ($\chi^2(7) = 35.926$, $p < 0.001$), and effects of time in both sham ($\chi^2(3) = 17.133$, $p = 0.001$) and SNL ($\chi^2(3) = 17.7$, $p = 0.001$) groups. The path length was also significantly decreased in trials 2–4 compared with trial 1 in both sham and SNL groups ($p < 0.01$) (Figure 3.23). These data suggest that both sham and SNL groups successfully recognised the platform as an escape route, and effectively learned to take the most direct path to the platform following repeated exposures. The swim speed was analysed by a
repeated-measures ANOVA, which revealed no significant effects. As such, SNL surgery did not appear to affect rats’ swimming ability (Figure 3.24). No other between-group differences were observed, suggesting SNL surgery did not affect any of the parameters measured in the cued trial.

Figure 3.22: Latency to get onto the platform in sham and SNL rats in the cued trial of the Morris water maze. The latency to get onto the platform decreased over trials in both groups. For ease of presentation, data are expressed as means ± SEM, n = 9 – 12. Wilcoxon signed-ranks test: ## p < 0.01 sham trials 2–4 vs. trial 1, ** p < 0.01 SNL trials 2–4 vs. trial 1.

The rats’ ability to learn the location of the hidden platform was assessed in the acquisition training phase. Again, both the latency to get onto the platform and the length of the path taken were analysed. The latency was analysed by Friedman’s ANOVA by ranks which revealed a significant overall effect ($\chi^2_{(9)} = 35.509$, $p < 0.001$) and significant effects of time in both sham ($\chi^2_{(4)} = 19.232$, $p = 0.001$) and SNL ($\chi^2_{(4)} = 24.318$, $p < 0.001$) groups. Wilcoxon signed-ranks tests showed that latency to get on to the platform decreased on days 3–5 compared with day 1 in the sham group ($p < 0.05$) and on days 2–5 compared with day 1 in the SNL group (Figure 3.25). The path length was analysed similarly, and a significant overall effect ($\chi^2_{(9)} = 42.77$, $p < 0.001$) and effects of time in both groups were found ($\chi^2_{(4)} = 21.511$, $p < 0.001$, sham; $\chi^2_{(4)} = 26.733$, $p < 0.001$, SNL).
Figure 3.23: Path length to locate the platform in sham and SNL rats in the cued trial of the Morris water maze. The path length decreased over trials in both groups. For ease of presentation, data are expressed as means ± SEM, n = 9 – 12. Wilcoxon signed-ranks test: ##p < 0.01 sham trials 2–4 vs. trial 1, **p < 0.01 SNL trials 2–4 vs. trial 1.

Figure 3.24: Swim speed of sham and SNL rats in the cued trial of the Morris water maze. For ease of presentation, data are expressed as means ± SEM, n = 9 – 12.

SNL). Path length was decreased on days 3–5 of acquisition compared with day 1 in the sham group and on days 2–5 compared with day 1 in the SNL group (p < 0.05) (Figure 3.26). These results suggest that both groups were able to learn the location of the platform according to the extra-maze visual cues over
the course of the training period. No differences were observed when comparing the performance of sham and SNL rats, indicating that there was no effect of SNL surgery on spatial reference learning. To rule out impaired swimming ability (or speed) as a confound of maze performance during the acquisition phase, the swim speed was also analysed throughout training. Repeated-measures ANOVA revealed a significant effect of time ($F_{(1,19)} = 30.391, p < 0.001$) but no effect of surgery or surgery $\times$ time interaction. There were no differences observed in the swim speed between nerve-ligated and control rats, suggesting SNL surgery did not affect swimming ability. There were significant decreases in swim speed on acquisition days 2–5 compared with the first day of training ($p \leq 0.001$) (Figure 3.27). Possible explanations for this decrease will be discussed in Section 3.4.

Figure 3.25: Latency to get onto the platform in sham and SNL rats in the acquisition phase of the Morris water maze. The latency to get onto the platform decreased over days in both groups. For ease of presentation, data are expressed as means $\pm$ SEM, $n = 9 – 12$. Wilcoxon signed-ranks test: $^# p < 0.05$, $^{##} p < 0.01$ sham days 3–5 vs. day 1, $^* p < 0.05$, $^{**} p < 0.01$ SNL days 2–5 vs. day 1.

The rats’ ability to recall the platform location was analysed in the probe trial. The % time spent in each of the four quadrant zones and in the platform and annulus zones was recorded, as well as the distance moved in each zone as a % of the total distance moved. It was expected that rats with intact spatial
Figure 3.26: Path length to locate the platform in sham and SNL rats in the acquisition phase of the Morris water maze. The path length decreased over days in both groups. For ease of presentation, data are expressed as means ± SEM, \( n = 9 - 12 \). Wilcoxon signed-ranks test: \(^\#p < 0.05\), \(^{##}p < 0.01\) sham days 3–5 vs. day 1, \(^*p < 0.05\), \(^{**}p < 0.01\) SNL days 2–5 vs. day 1.

Figure 3.27: Swim speed of sham and SNL rats in the acquisition phase of the Morris water maze. The swim speed decreased over days of testing in both groups. Data are presented as means ± SEM, \( n = 9 - 12 \). Fishers’ LSD post hoc analysis: \(^{###}p < 0.001\) sham days 2–5 vs. day 1, \(^{***}p < 0.001\) SNL days 2–5 vs. day 1.
memory would show a preference for the southwest quadrant where the platform had previously been located. However, the % time spent and the % distance moved in the southwest quadrant were close to the level of chance (25%) in both sham and SNL groups, suggesting that spatial memory was impaired to some degree in both treatment groups, a finding which is discussed in Section 3.4. There were no differences between sham and SNL groups in either measure (% time or % distance moved) for any of the zones analysed, which suggests that SNL surgery did not have any direct effects on spatial memory (Figures 3.28, 3.29). There were no effects of SNL surgery on total distance moved or swim speed in the probe trial (Figures 3.30, 3.31), as had been observed in both the cued trial and the acquisition training phase.

Figure 3.28: % time spent in arena zones during the probe trial in sham and SNL groups. Data are presented as means ± SEM, n = 9 – 12. The dashed line represents the % of time animals would spend in each quadrant by chance (25%).
Figure 3.29: % distance moved in arena zones during the probe trial in sham and SNL groups. Data are presented as means ± SEM, n = 9 − 12. The dashed line represents the % distance animals would move in each quadrant by chance (25%).

Figure 3.30: Total distance moved by sham and SNL rats during the probe trial. Data are presented as means ± SEM, n = 9 − 12.
3.3.3 IB4 staining

A qualitative decrease in IB4 staining was observed in the dorsal horn of the spinal cord at the level of L5 and L6. Staining was unaffected at the L4 level (Figure 3.32). Following quantification of IB4 staining intensity using ImageJ software, two-way ANOVA showed significant main effects of level ($F_{(2,57)} = 5.92, p = 0.005$), group ($F_{(1,57)} = 61.0, p < 0.001$) and level × group interaction ($F_{(1,57)} = 9.50, p < 0.01$). The intensity was decreased selectively in the L5 and L6 regions in SNLs compared with the corresponding levels in sham controls (***$p < 0.001$, Fisher’s LSD post hoc analysis), and compared with the L4 level in SNLs (###$p < 0.001$, Fisher’s LSD post hoc analysis) (Figure 3.33 (a)). The IB4 intensity at the L5 and L6 levels was positively correlated with the mechanical response thresholds measured in the von Frey test (Figure 3.33 (b) and (c)). The depletion of central terminals of IB4-positive cells has been shown previously in models of neuropathic pain, and indicates a disruption of sensory information transmission in the spinal cord following SNL surgery.
Figure 3.32: (i)–(iii) Reference images of L4–L6 transverse sections from the Rat Brain Atlas (Paxinos and Watson, 1998). (iv)–(vi) Representative images of IB4-stained sections from regions L4–L6 of the spinal cord of sham control rats. (vii)–(ix) Representative images of IB4-stained sections from regions L4–L6 of the spinal cord of SNL rats. SNL-operated rats showed a qualitative decrease in IB4 staining intensity in laminae I–II of the dorsal horn of the spinal cord on the ipsilateral side in the regions corresponding to the sites of ligation (L5 and L6), compared with both the contralateral side, and compared with both sides in the sham controls. Scale bar = 1mm.
Figure 3.33: Quantification of IB4 staining intensity in the ipsilateral dorsal horn of the spinal cord as a percentage of expression on the contralateral side. (a) There was no effect of SNL surgery on the intensity of IB4 staining at the L4 level. IB4 staining intensity was decreased in the SNL group compared with the sham group at both the L5 and L6 levels (**p < 0.001) and expression was also lower in these regions than at the L4 level in the same animals in the SNL group (###p < 0.001). Data are presented as means ± SEM. n = 9 - 12. (b) and (c) There was a significant positive correlation between the mechanical response threshold (measured in the von Frey test) and the intensity of IB4 staining at both the L5 (r = 0.59, p = 0.005) and L6 (r = 0.70, p < 0.001) levels. Each plotted point corresponds to an individual animal (sham or SNL).
3.4 Discussion

The SNL model was found to be associated with characteristic neuropathic pain-like behaviours, mechanical allodynia and thermal hyperalgesia at discrete time points relative to surgery. Both between-group comparisons of ipsilateral responses in sham and SNL groups and within-subject comparisons of ipsilateral and contralateral paws in the SNL group showed that the surgery was associated with increased sensitivity of the ipsilateral hindpaw to mechanical stimuli. There was no effect of surgery on the responses in the contralateral paw. The finding of a decrease in mechanical threshold (and an increase in the % response) in both paws in the sham controls and in the contralateral paw of SNL rats over time was not anticipated and is not commonly observed in the literature. It is unlikely that this decrease was as a consequence of the sham surgery as it persisted throughout the test period, up to 13 days post surgery. In addition, a similar decrease in mechanical threshold over time is described in a later chapter (Chapter 4) in intraplantar needle insertion control rats that did not undergo sham SNL surgery. Moreover, we do not believe the difference to be caused by experimenter bias, as the tester was blind to the surgical treatment of each animal. The decrease in threshold in all subsequent test sessions compared with the first session appears to be an artefact of our test procedure, which, as described in Chapter 2, is a modification of a number of commonly used von Frey methods. Our method involves the application of a relatively high number of stimuli (five applications of each filament per paw, up to 14 filaments). Therefore, it is possible that the rats became sensitised to the application of filaments over time and thus showed an enhanced response. This hypothesis is consistent with the observation by (Chaplan et al., 1994) that repetitive low-intensity stimulation of the hindpaw of naïve unoperated rats was associated with a decrease in 50% response threshold over time, presumed to be an adaption of behaviour in response to “annoyance or nuisance in the absence of nociception”. The absence of such a decrease in the ipsilateral paw of nerve-ligated rats is presumably due to a “ceiling effect”, in that...
thresholds in this group were already significantly lower than controls on day 1 of testing. When ipsilateral and contralateral responses in shams were compared (Figure 3.6), a significant decrease in threshold was observed in the ipsilateral paw on day 9 post surgery. This finding may demonstrate a degree of experimenter bias because, despite being blinded to the treatment of each rat, the experimenter was aware of the side of the body on which the surgery was performed. However, if measurements were subject to this type of bias, a significant difference would be expected throughout the observation period and this was not the case. Another possibility is that the sham surgery was associated with minor non-specific damage to the sensory nerves and the effect was only manifested at this time point (day 9). In the Hargreaves test, a decrease in latency to respond to a thermal stimulus was observed, both in the SNL ipsilateral paw compared with the sham ipsilateral paw and when the SNL ipsilateral paw response was compared with the SNL contralateral paw response. No changes in paw withdrawal latency were observed in the contralateral paw. Experiments measuring hypersensitivity in peripheral nerve injury models commonly apply a minimum threshold reduction as an inclusion criterion (for example, 80% of baseline). Such a criterion could not be applied in this experiment as baseline sensory thresholds were not measured. There did not appear to be a strong correlation between post-surgical sensory thresholds and cognitive measures, and as such, all animals that had undergone SNL surgery were included in the analysis of cognitive performance.

IB4 histochemical staining was reduced in laminae 1–2 of the ipsilateral dorsal horn of SNL rats compared with sham controls. This has been shown previously in SNL (Bennett et al., 2000; Pezet et al., 2006) and in other models of neuropathic pain (Munglani et al., 1995). This decrease is thought to reflect damage to the IB4-positive subpopulation of C-fibre nociceptors (Silverman and Kruger, 1988) and suggests the ligation caused injury at the level of the spinal cord. Notably, the decrease in IB4 staining occurred at the L5 and L6 levels but not at the level of L4, confirming the damage was site-specific to the ligated spinal nerves.
In the novel object recognition task, neither sham nor SNL rats showed a preference for either object in Exposure 1, but both groups displayed a preference for the novel object in Exposure 2. This result suggests that both groups were able to discriminate between the novel and familiar objects, indicating intact recognition memory. Our results did, however, indicate a trend for decreased novel object discrimination in the SNL group compared with the sham group. It is possible that this observation reflects mild impairment of recognition memory in this group. Importantly, the observed difference was not as a result of motoric effects of the SNL surgery. We found that the SNL group did not differ from the sham controls on measures of locomotor activity or other exploratory behaviours, similar to observations by others (Kim and Chung, 1992; Chung et al., 2004). There were no effects of SNL surgery on performance of animals in the T-maze spontaneous alternation task. In the Morris water maze, escape latency and path length decreased over repeated trials in the cued task, suggesting that rats had accurate visual perception, sufficient motivation to try to escape from the maze, and recognition of the platform as the escape route. There were no differences between sham and SNL groups. Swim speed was also unchanged between groups and did not change over time in the cued task. A similar pattern was observed in the acquisition training phase: latency and path length to the platform decreased over time, which demonstrates that rats learned the position of the platform over time with reference to the spatial cues. The rate of learning was similar in sham and SNL groups, and there was no difference between the groups on any of the test days. During acquisition, swim speed was also found to decrease over repeated days of testing in both sham and SNL groups. This was somewhat unexpected given that the latency to get onto the platform decreased. However, it is possible that following repeated exposure to the maze, animals were habituated to the environment and were therefore less likely to explore, resulting in a decrease in swim speed. A similar decrease in locomotor activity has been observed in naïve rats following repeated exposure to an open field environment (Whishaw
et al., 2006). The probe trial was used to measure spatial memory. We found that neither sham nor SNL groups showed a significant preference for the southwest quadrant of the maze, in which the platform had been located. The time spent in the southwest was close to the level of chance and this suggests that neither group could effectively recall the platform location. The reason for this is unclear. It is possible that reference memory was impaired in both groups; however, this seems improbable given that rats performed well during the acquisition phase. A limitation of water maze testing in this experiment is the small diameter of the water maze used (90 cm). This size maze is more commonly used for Morris water maze testing in mice. Based on the size of the rat relative to the arena, it is possible that the animal may have been reasonably close to the platform, but not in the target quadrant. This may account for the lack of discrimination for the platform quadrant in the probe trial. There were no differences between sham and SNL groups in the probe trial and SNL surgery did not affect swim speed or the total distance moved during the trial. The detection of mild cognitive impairments in the novel object recognition test and the failure to detect impairment in the water maze may arise from differences in the degree of aversiveness or stress associated with the individual tasks. Exposure to the water maze may evoke a stress response which may, in turn, activate descending endogenous analgesic mechanisms (stress-induced analgesia, for review see Butler and Finn (2009)), thereby reducing the impact of pain on cognitive function. The absence of aversive ultrasounding in the novel object paradigm suggests that that paradigm was not associated with a stress/aversive response. However, distraction, including that caused by exposure to a novel object, has also been proposed as form of endogenous analgesia (Ford et al., 2008). Spontaneous pain behaviour in the SNL model is uncommon, and cognitive deficits observed in chronic pain are thought to involve adaptive neural changes (see Chapter 1). Therefore, it is unlikely that modulation of acute pain by stress or distraction affected cognition during the behavioural tests. The motivational state of the animal may also have affected cognitive task performance.
Depressive-like symptoms have been demonstrated previously in animal models of chronic pain (Hu et al., 2010; Leite-Almeida et al., 2009; Shi et al., 2010a; Suzuki et al., 2007), and thus decreased motivation to complete the cognitive tasks might have been expected in SNL rats in the present study. However, such an effect is not indicated by the present data. Another possible explanation for the observation of deficits in the novel object paradigm, but not in the water maze, is that different cognitive domains are differentially affected in the SNL model, with recognition memory more susceptible to impairment than spatial learning and memory.

As mentioned previously, early research did not address potential cognitive deficits in an animal model of neuropathic pain. Impairments in attention and in spatial and recognition memory had been demonstrated in inflammatory pain models (Boyette-Davis et al., 2008; Cain et al., 1997; Lindner et al., 1999; Millecamps et al., 2004). However, more recent studies have investigated cognitive function in animal models of neuropathic pain. It was found that rats that had undergone spared nerve injury or L5 transection had impaired spatial learning and memory in the Morris water maze (Hu et al., 2010; Leite-Almeida et al., 2009), and impaired working and reference memory in a radial arm maze (Ren et al., 2011). In the light of these findings, the results of the present study are surprising, as we did not find any pain-related deficits in spatial, reference or working memory, or in spatial learning. This discrepancy may be explained by differences in the pain model used, by the use of different cognitive measures or by subtle differences in protocols employed where the same tests were used. Significantly, the study by Leite-Almeida et al. (2009) found cognitive deficits associated with neuropathic pain specifically in a mid-aged cohort of rats, but not in young or old rats. These results highlight age as an important factor in the expression of pain-related cognitive impairment. Differences between age cohorts may arise as a result of a variety of age-related alterations in neural plasticity, neurotransmitter and neuromodulator levels, structural changes in the brain and neuroendocrine alterations. It is possible, therefore, that we were unable to detect cognitive changes
associated with SNL surgery because young adult rats (aged 6–9 weeks on arrival) were used in the present experiment. This issue is addressed experimentally in later chapters.

As this study represented an initial characterisation of neuropathic pain-like behaviours and cognitive function in the SNL model, a number of experimental limitations should be noted. No baseline measurements of sensory responses in the von Frey and Hargreaves tests were carried out and so pre- and post- surgery comparisons of mechanical and thermal sensitivity cannot be made. There was a long time interval between the first von Frey test session, on day 1 post surgery, and the second, on day 9 post surgery. The expression of mechanical allodynia was subtle and was only present at certain post-surgical time points. In von Frey testing, there was no difference in the 50% withdrawal threshold between sham and SNL groups on the last day of testing, although the SNL rats were hyperresponsive to the 6 g von Frey filament. It would also be preferable to include additional test days for thermal hyperalgesia, such that the temporal development of thermal hypersensitivity could be monitored. The increased sensitivity of sham controls to mechanical stimuli suggests that the control group may have also developed increased sensitivity to mechanical and thermal stimuli, which would have compromised the ability to detect a difference in cognition between SNL rats and sham controls. However, as discussed earlier in the section, this decrease in response threshold in the sham group is thought to represent merely a behavioural sensitisation to the test protocol. The potential consequences of using the small-diameter maze for Morris water maze testing are also detailed earlier in the section. A number of these limitations are addressed in later chapters.

Despite the noted limitations, and the largely negative findings with respect to effects of neuropathic pain behaviour on cognition, a number of important objectives were met. The surgical SNL technique was successfully established and replicated, and was found to be associated with characteristic pain-like behaviours at discrete time points and decreased IB4 staining in the ipsilateral spinal cord.
Protocols for von Frey and Hargreaves testing, as well as the cognitive behavioural tests, were established. There was a trend for impaired novel object recognition in the SNL group; however, this failed to reach the level of statistical significance. No other alterations in cognitive function were observed in the SNL model under the experimental conditions used.
4.1 Introduction

In Chapter 3, no effects of SNL surgery (as a model of neuropathic pain) on cognition in young adult male rats in a variety of commonly used behavioural tests measuring spatial and recognition memory were found. This is despite convincing evidence for the existence of cognitive impairments in clinical pain populations, as outlined in Chapter 1. Thus, we hypothesised that our initial experiments (Chapter 3) involved either inappropriate assessments of cognition, or an inappropriate model of clinical chronic pain. In order to address the first of these issues, attempts were made to investigate the effects of chronic pain on a different type of cognitive behaviour – aversive learning and memory. Regarding the second issue, a second pain model – intraplantar injection of Complete Freund’s Adjuvant (CFA) – was also investigated in this chapter. CFA is used to model chronic inflammatory pain.

There is a substantial overlap in the neuroanatomical regions that mediate aversive learning/memory and pain. These include limbic regions such the amygdala and hippocampus (Ambrogi Lorenzini et al., 1997; Apkarian et al., 2005; LeDoux, 1993; Lorenzini et al., 1996). Moreover, chronic pain has been likened to the continuous presence of an unconditioned aversive stimulus, which can become associated with random conditioned events and inhibit extinction of associative memory on re-exposure to the conditioned events (Apkarian, 2008). Therefore, it may be postulated that chronic pain would negatively affect associative learning.
for other aversive stimuli.

While the influence of conditioned aversive or stress responses on pain levels has been well studied (for reviews, see Ford and Finn 2008; Butler and Finn 2009), there is a paucity of studies investigating the effect of pain on recall of aversive/stressful memories. However, research has shown that reporting of previous pain is distorted in chronic pain patients (Bryant, 1993; Eich et al., 1985). In contrast, other studies have shown that both clinical and experimental pain can enhance recall of aversive words in a memory task (Pearce et al., 1990; Seltzer and Yarczower, 1991), and increased pain levels are associated with increased re-experiencing symptoms in post-traumatic stress disorder (Beckham et al., 1997), suggesting that pain may in fact enhance aversive memory. Experimental pain was also found to affect context-dependent memory, though this effect was significant only for neutral cues, and not for aversive cues (Schwabe et al., 2009). In animal models of pain, cognitive impairments have been demonstrated previously in behavioural paradigms including novel-object exploration, operant learning tasks and the Morris water maze tests of learning and memory (Boyette-Davis et al., 2008; Hu et al., 2010; Leite-Almeida et al., 2009; Millecamps et al., 2004; Pais-Vieira et al., 2009a). However, as is the case for the clinical research, there are few studies specifically investigating aversive learning and memory in animal models of chronic pain. Studies have shown that rats can acquire an avoidance response in place escape/avoidance paradigms following context-specific, aversive stimulation of the injured hindpaw in models of chronic pain (LaBuda and Fuchs, 2000; Pedersen and Blackburn-Munro, 2006). A study by Suzuki et al. (2007) found that spinal nerve ligation did not affect aversive learning in a foot-shock induced passive-avoidance paradigm, and Mutso et al. (2012) recently demonstrated that rats that had undergone SNI surgery as a model of neuropathic pain could acquire a conditioned fear response to foot-shock. In these cases, however, the aversive stimuli are noxious in nature and are applied to the same site as the chronic pain model injury. To our knowledge, there are no studies that have assessed aversive
learning induced by a non-painful, sensory, aversive conditioning stimulus in a model of chronic pain.

Passive avoidance is a paradigm used to measure short-term or long-term aversive memory in laboratory rodents. It is a fear-motivated classical conditioning paradigm, in which rodents learn to associate a conditioned stimulus or context with an aversive or noxious stimulus, resulting in the subsequent avoidance of the conditioned stimulus. Simple passive-avoidance paradigms are generally carried out in an arena with light and dark compartments and in their simplest form involve a single acquisition trial, during which an instinctive response (moving from the light to the dark compartment) is punished by administration of an aversive stimulus in the dark compartment. The aversive stimulus most commonly used is noxious foot-shock. After a specified interval, the animal is returned to the arena (retention trial) and avoidance of the punished (dark) context is observed. This traditional method of foot-shock-induced passive avoidance is, however, complicated in models of chronic pain, as they are commonly associated with hypersensitivity of the hindpaw(s). Because foot-shock stimulates peripheral nociceptors in the paws, the stimulus is likely to be perceived differently in models of chronic pain, which may well confound interpretation of results with respect to cognition. We therefore investigated a number of aversive stimuli for their suitability as non-noxious alternatives to foot-shock in a passive-avoidance paradigm designed to assess aversive memory in rodent models of chronic pain.

In stressful situations, including exposure to a predator, attack by a conspecific, or shock, rats emit ultrasonic vocalisations in the range of 18–27 kHz (Blanchard et al., 1991; Sales, 1972). Exposure to artificially generated ultrasound at a frequency of 20 kHz is associated with a defensive or aversive behavioural response which is characterised by bouts of hyperlocomotion and freezing (Beckett et al., 1996; Commissaris et al., 2000; Finn et al., 2004), as well as increases in neurochemical markers of aversion including increased expression of c-Fos in the PAG (Beckett et al., 1997). Ultrasound was shown previously to induce avoidance be-
haviour in a passive-avoidance paradigm (Dokla et al., 1989). A brief exposure to compressed air (“air-puff”) applied to the head of rodents can induce fear-related behaviour such as a startle response (Engelmann et al., 1996) and 22 kHz ultrasonic vocalisation (Knapp and Pohorecky, 1995; Sanchez, 2003). Conditioning to aversive air-puff has also been shown to evoke avoidance behaviour (Cimadevilla et al., 2001; Karlsson et al., 2009; Koistinaho et al., 2001).

The aims of the present experiment were to test both ultrasound and air-puff as aversive stimuli in a passive-avoidance paradigm, to validate the preferred stimulus in a passive-avoidance paradigm using the amnesic drug scopolamine (Figure 4.1), and to use this validated paradigm to assess aversive learning and memory in two rat models of chronic pain: the SNL model of neuropathic pain and the CFA model of inflammatory pain.

4.2 Methods

4.2.1 Animals

Young adult male Sprague-Dawley rats were used in all of the experiments described in this chapter. Rats were 6–9 weeks old on arrival at our facility and weighed 175–250 g. As described in Chapter 2, experimental procedures were conducted with the approval of appropriate agencies and according to relevant ethical guidelines. Animals were housed under standard conditions of light and temperature, and food and water were available ad libitum.
4.2.2 Experimental protocols

A detailed description of the apparatus and test procedure for the use of both ultrasound and air-puff in the passive avoidance paradigm is provided in Chapter 2.

**Experiment 1: Use of ultrasound in the passive-avoidance paradigm**

Rats were habituated to the facility for a period of 5 days prior to testing. They were randomly assigned to either ultrasound \( n = 16 \) or no-ultrasound \( n = 8 \) groups. Acquisition and retention trials were carried out as described in Chapter 2. No pre-acquisition habituation phase was included in this experiment. The latency to enter the dark compartment, known as the “step-through latency” was recorded for both trials.

**Experiment 2: Use of air-puff in the passive-avoidance paradigm and pharmacological validation of the response using scopolamine**

Rats were habituated to the facility for a period of 6 days prior to testing. In addition, rats were habituated to i.p. injections of physiological saline (0.9%) for a period of 4 days prior to testing to minimise injection-related stress. In the air-puff passive avoidance paradigm, an habituation period of 5 minutes, in which rats were allowed to freely explore both light and dark compartments of the arena, was included on the day before acquisition training. As described in Chapter 2, this was included to minimise anxiety induced by introduction to a novel arena. Rats were then divided into air-puff \( n = 24 \) and no-air-puff \( n = 8 \) groups. No-air-puff rats received a single i.p. dose of physiological saline 30 minutes before the acquisition trial. Air-puff rats were subdivided into saline \( n = 8 \) or scopolamine hydrobromide at a dose of 1 mg/kg \( n = 8 \) or 3 mg/kg \( n = 8 \), also administered i.p. 30 minutes prior to acquisition. Rats underwent acquisition and retention trials as described in Chapter 2 and step-through latencies were recorded. To investigate the extinction of the air-puff-induced passive-avoidance response, a separate cohort of rats \( n = 7 \) were subjected to daily repeated
retention trials for 5 days, where no additional aversive stimuli were applied, and the step-through latency was recorded daily.

**Experiment 3: Assessment of aversive learning in rat models of chronic neuropathic and inflammatory pain**

Baseline sensory testing began a minimum of 3 days following arrival of rats to the facility. The SNL model of neuropathic pain and the CFA model of inflammatory pain were induced according to the methods described in Chapter 2. There were four experimental groups: SNL \((n = 11)\), sham control surgery \((n = 12)\), CFA \((n = 9)\) and needle-insertion control \((n = 9)\) groups. von Frey, Hargreaves and acetone-drop tests were used to assess mechanical allodynia, thermal hyperalgesia and cold allodynia, respectively, as described in Chapter 2. von Frey testing was carried out 2–4 days prior to SNL surgery or CFA injection, and on days 1, 3, 5, 7, 9, 11 and 13 post injury. The arena used was 15 cm \((h)\) × 11 cm \((l)\) × 20 cm \((w)\), and was separated from the adjoining compartment by a Perspex partition. Hargreaves testing was carried out 1–3 days prior to SNL surgery or CFA injection, and on days 8 and 16 post injury. Testing for cold allodynia was carried out 3–5 days prior to SNL surgery or CFA injection, and on days 4 and 12 post injury. Hindpaw oedema following injection of CFA was assessed by measuring paw volume and paw circumference pre injection and post injection. Procedures are outlined in Chapter 2. Levels of IB4 in the dorsal horn of the spinal cord were qualitatively measured, also as described in Chapter 2. Following surgery, nerve-ligated animals and sham controls were subdivided into air-puff and no-air-puff group (SNL air-puff: \(n = 6\), SNL no-air-puff: \(n = 5\), sham air-puff: \(n = 6\), and sham no-air-puff: \(n = 6\)). All CFA-treated rats and controls were exposed to air-puff (CFA air-puff \(n = 9\) and control air-puff \(n = 9\)). Passive-avoidance testing was carried out as described above on days 18 (habituation), 19 (acquisition) and 20 (retention) post SNL surgery or post CFA injection.

The timelines for the three experiments are illustrated in Figure 4.2.
Figure 4.2: Timelines for the three experiments described in this chapter.
4.2.3 Statistical analysis

All data were tested for normality and for homogeneity of variance, using Shapiro-Wilk and Levene’s tests, respectively. Parametric data were analysed using two-way repeated-measures ANOVA or by a one-way ANOVA. Fisher’s least-significant-difference (LSD) or Student-Newman-Keuls (SNK) tests were used to make post hoc comparisons, as appropriate. Where possible, non-parametric data were square-root or log transformed and analysed similarly to parametric data. If non-parametric data could not be transformed, they were analysed using Friedman’s two-way ANOVA by ranks, followed by Mann-Whitney U or Wilcoxon signed-ranks tests, and $p < 0.05$ was considered statistically significant. Data were analysed using SPSS software for Windows and results were depicted graphically with the aid of GraphPad Prism® software. For clarity of presentation, data are expressed as means ± SEM, with the exception of Experiment 1 data; due to a large difference in experimental subject number between groups in Experiment 1, these data are expressed as means ± standard deviation (SD) (Figure 4.3).

4.3 Results

4.3.1 Experiment 1: Use of ultrasound in the passive-avoidance paradigm

Delivery of aversive ultrasound was tested for its ability to produce a passive-avoidance response. Exposure to ultrasound (frequency 21 kHz, level 100 ± 10 dB, duration 1 minute) did not result in any significant difference in step-through latency between the acquisition and retention trials, suggesting that it did not cause a passive-avoidance response (Figure 4.3).

4.3.2 Experiment 2: Use of air-puff in the passive-avoidance paradigm and pharmacological validation of the response using scopolamine

In the air-puff paradigm, Friedman’s ANOVA by ranks revealed an overall effect ($\chi^2_{(7)} = 32.225$, $p < 0.001$). There was also a significant effect of group,
Figure 4.3: Effect of ultrasound exposure on behaviour in the light/dark arena. Exposure of rats to 21 kHz ultrasound was not associated with a passive-avoidance response. There were no differences between acquisition and retention step-through latencies, and no differences between the ultrasound \((n = 15)\) and no-ultrasound \((n = 7)\) groups. Due to the difference in the number of experimental subjects in each group, data are presented as mean ± standard deviation (SD).

based on a Kruskal-Wallis test \((\chi^2_{(3)} = 9.696, p = 0.021)\). Step-through latency during the acquisition trial did not differ between the air-puff saline, air-puff scopolamine 1 mg/kg or 3mg/kg and no-air-puff groups (Mann-Whitney \(U\) tests \(p > 0.05\); Figure 4.4). Wilcoxon tests showed that air-puff produced a significant passive-avoidance response in the air-puff saline and in the air-puff scopolamine 3 mg/kg group \((^{*}p < 0.05\) retention vs. acquisition\), and a trend for a passive-avoidance response in the air-puff scopolamine 1 mg/kg group \((p = 0.09)\).

There was no passive-avoidance response in the no-air-puff saline group, indicating that the avoidance response was a specific consequence of prior exposure to air-puff. Mann-Whitney \(U\) tests revealed that the latency in the retention trial was significantly greater in the air-puff saline group than in the no-air-puff saline group \((^{+}p = 0.001)\). The retention-trial step-through latency in the scopolamine 3 mg/kg group was significantly shorter than that of the air-puff saline group \((^{#}p < 0.01, \text{Mann-Whitney} \ U\ \text{test}; \ \text{Figure} \ 4.4)\). To investigate the extinction profile of the air-puff-induced passive-avoidance response, a separate cohort of rats was tested in repeated retention trials over 5 days. One-way ANOVA of log-transformed data revealed a significant effect \(F_{(6,48)} = 5.288, p < 0.001)\).
Step-through latency was increased in the retention trial and subsequent extinction trials ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$), indicating that the avoidance response was still evident after 5 days of extinction training (Figure 4.5).

Figure 4.4: Passive-avoidance response induced by air-puff, and effect of scopolamine. Step-through latency during the acquisition trial did not differ between the experimental groups. Saline-treated rats exposed to air-puff exhibited a significant passive-avoidance response, measured as a significant increase in the step-through latency in the retention trial compared with the acquisition trial ($*p < 0.05$, saline air-puff, retention vs. acquisition). There was no increase in step-through latency in saline-treated rats not exposed to air-puff. Retention step-through latency in saline-treated rats exposed to air-puff was significantly greater than in saline-treated rats not exposed to air-puff ($^{+}p = 0.001$). Scopolamine-treated rats exhibited an increased step-though latency during the retention trial relative to the acquisition trial ($^*p < 0.05$, scopolamine 3 mg/kg air-puff, retention vs. acquisition) but the magnitude of the increase was less than that observed in saline-treated rats. Scopolamine treatment resulted in a dose-related partial attenuation of the passive-avoidance response, with a significant effect at the 3 mg/kg dose ($^{#}p < 0.01$, air-puff scopolamine 3mg/kg vs. air-puff saline). Data are expressed as means ± SEM. $n = 8$ per group.
4.3.3 Experiment 3: Assessment of aversive learning in rat models of chronic neuropathic and inflammatory pain

4.3.3.1 Sensory testing

The development of chronic pain-like behaviours was investigated using von Frey, Hargreaves and acetone-drop tests. Withdrawal threshold to mechanical stimulation of the ipsilateral hindpaw of SNL rats was compared with that of the ipsilateral paw of sham control rats in the von Frey test. Friedman’s ANOVA by ranks revealed an overall effect ($\chi^2(15) = 89.231$, $p < 0.001$) and significant effects of time in both sham ($\chi^2(7) = 30.91$, $p < 0.001$) and SNL ($\chi^2(7) = 29.312$, $p < 0.001$) groups. Wilcoxon signed-ranks tests revealed that response thresholds were lower at all post-surgery time points in both the sham group ($^{++}p < 0.01$) and the SNL group ($^{##}p < 0.01$) compared with their respective baselines (Figure 4.6). Mann-Whitney $U$ tests revealed that thresholds were significantly lower in SNL group than in the sham control group at all post-SNL time points ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Similarly, the mechanical threshold of the CFA-injected hindpaw was compared
Figure 4.6: Effect of SNL surgery on the mechanical response threshold of the ipsilateral hindpaw. SNL rats expressed mechanical allodynia at all post-surgery time points compared with sham controls and with pre-surgery baseline. There was also a decrease in threshold in the sham controls post-surgery compared with baseline. Mann-Whitney U tests: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ SNL vs. sham; Wilcoxon signed-ranks test: ++$p < 0.01$ sham post surgery vs. baseline, ##$p < 0.01$ SNL post surgery vs. baseline. Data are expressed as means ± SEM. $n = 11 - 12$.

to that of the needle-inserted control paw. Friedman’s ANOVA by ranks revealed an overall effect ($\chi^2_{(15)} = 117.554, p < 0.001$) and effects of time in both control ($\chi^2_{(7)} = 39.462, p < 0.001$) and CFA ($\chi^2_{(7)} = 34.294, p < 0.001$) groups. Wilcoxon signed-ranks tests revealed that response thresholds were lower at all post-injection time points in both the control (++$p < 0.01$) and the CFA (##$p < 0.01$) groups compared with respective their baselines. Mann-Whitney U tests revealed that thresholds were significantly lower in the CFA group than in the control group at all post-injection time points (***$p < 0.001$) (Figure 4.7). As with previous experiments, the % withdrawal responses to specific von Frey filaments and the threshold responses in the contralateral hindpaws were also measured. These results are presented in Appendix B, Section B.1.

In the Hargreaves test, SNL-operated rats were found to express thermal hyperalgesia in the hindpaw ipsilateral to ligation. Two-way repeated-measures
Figure 4.7: Effect of CFA injection on the mechanical response threshold of the ipsilateral hindpaw. CFA-treated rats expressed mechanical allodynia at all post-injection time points compared with sham controls and with pre-injection baseline. There was also a decrease in threshold in the control rats post treatment (needle-insertion) compared with baseline. Mann-Whitney U tests: ***$p < 0.001$ CFA vs. control; Wilcoxon signed-ranks test: ##$p < 0.01$ CFA post injection vs. baseline, ++$p < 0.01$ control post treatment vs. baseline. Data are expressed as means ± SEM. $n = 9$.

ANOVA of square-root transformed data revealed an overall effect of time ($F_{(2,42)} = 4.240$, $p < 0.05$), surgery group ($F_{(1,21)} = 10.923$, $p < 0.01$) and surgery × time interaction ($F_{(2,42)} = 4.849$, $p < 0.05$). SNK post hoc tests revealed a decrease in the ipsilateral hindpaw withdrawal latency to thermal stimulation in the SNL group at all post-surgery time points compared with baseline (##$p < 0.01$). The SNL group also had a shorter response latency than the sham control groups at all post-SNL time points (*$p < 0.05$, **$p < 0.01$, Figure 4.8).

CFA-treated rats were also found to express thermal hyperalgesia of the injected hindpaw. Two-way repeated-measures ANOVA revealed an overall effect of time ($F_{(2,42)} = 24.352$, $p < 0.001$), treatment ($F_{(1,21)} = 23.771$, $p < 0.001$) and time × treatment interaction ($F_{(2,42)} = 6.897$, $p < 0.01$). SNK post hoc tests revealed a decrease in the latency to respond to thermal stimulation post
Figures 4.8 and 4.9: Effect of SNL surgery and CFA injection on ipsilateral hindpaw withdrawal latency in the Hargreaves test. Paw withdrawal latency was decreased in the ipsilateral hindpaw of SNL and CFA rats compared with sham and control rats on days 8 and 16 post surgery, and compared with baseline. SNK post hoc tests: *p < 0.05, **p < 0.01, SNL vs. sham control; ###p < 0.01, SNL post surgery vs. baseline. Data are expressed as means ± SEM. n = 11–12.

Data for Figure 4.8: sham control (black circles), SNL (black squares). Data for Figure 4.9: control (open circles), CFA (black squares).

Figure 4.8: Effect of SNL surgery on ipsilateral hindpaw withdrawal latency in the Hargreaves test. Paw withdrawal latency was decreased in the ipsilateral hindpaw of SNL rats compared with sham rats on days 8 and 16 post surgery, and compared with baseline. SNK post hoc tests: *p < 0.05, **p < 0.01, SNL vs. sham control; ###p < 0.01, SNL post surgery vs. baseline. Data are expressed as means ± SEM. n = 11–12.

Figure 4.9: Effect of CFA injection on withdrawal latency of the ipsilateral hindpaw in the Hargreaves test. Paw withdrawal latency was decreased in the ipsilateral paw of CFA rats compared with controls rats on days 8 and 15 post surgery, and compared with baseline. SNK post hoc tests: ***p < 0.001, CFA vs. control; ###p < 0.01, CFA post injection vs. baseline. Data are expressed as means ± SEM. n = 9.
injection compared with baseline (**p < 0.05). The CFA group had a decreased response latency compared with the control group at all post-injection time points (****p < 0.001, Figure 4.9). Paw withdrawal latencies for the contralateral paw are presented in Appendix B, Section B.1.

The acetone-drop test was used to assess the development of cold allodynia of the ipsilateral hindpaw of SNL-operated and CFA-treated rats. For SNL and sham controls, two-way repeated-measures ANOVA of square-root transformed data revealed an overall effect of time ($F_{(2,42)} = 63.027$, $p < 0.001$), surgery group ($F_{(1,21)} = 9.810$, $p < 0.01$) and surgery $\times$ time interaction ($F_{(2,42)} = 3.524$, $p < 0.05$). Post hoc tests revealed an increase in the total number of responses to acetone application post surgery in both the sham (+++p < 0.001) and SNL (###p < 0.05) groups, compared with their respective baselines. Nerve-injured rats had a significantly greater number of responses than the sham control group at all post-surgery time points (**p < 0.01) (Figure 4.10).
Figure 4.11: Effect of CFA injection on the total number of hindpaw withdrawal responses following acetone application to the ipsilateral paw. The number of ipsilateral hindpaw withdrawal responses to acetone was significantly greater in CFA rats compared with sham controls at all post-injection time points, and compared with baseline. There was also a significant increase in the number of responses to acetone in the control group post treatment compared with baseline. SNK post hoc tests: **p < 0.01, CFA vs. control; #p < 0.05, CFA post injection vs. baseline. Data are expressed as means ± SEM. n = 9.

Similar effects were observed following the injection of CFA. Two-way repeated-measures ANOVA of square-root transformed data revealed an overall effect of time ($F_{(2,42)} = 16.434, p < 0.001$), treatment ($F_{(1,21)} = 9.858, p < 0.01$) and time × treatment interaction ($F_{(1,21)} = 5.135, p < 0.05$). SNK post hoc tests revealed an increase in the total number of responses to acetone application post injection in the CFA group but not in the control group (#p < 0.05) (Figure 4.11). There was a significantly greater number of responses in the CFA group compared with the control group at all post-injection time points (**)p < 0.01). Results of the acetone-drop test for the contralateral paw are presented in Appendix B.

CFA injection was associated with a greater increase in paw volume than that observed in needle-insertion controls. The relative change in paw volume between pre-injection and post-injection measurements was significantly greater in the CFA group than in the control group (***p < 0.001, Student’s unpaired t-test, Figure 4.12). CFA injection was also associated with a greater increase in paw circumference than that observed in needle-insertion controls. Relative change
in paw circumference between pre-injection and post-injection measurements was significantly greater in the CFA group than in the control group (***(p = 0.001, Student’s unpaired t-test, Figure 4.13).
4.3.3.2 **IB4 staining**

SNL-operated rats showed a qualitative decrease in IB4 staining intensity in laminae I-II of the dorsal horn of the spinal cord on the ipsilateral side in the regions corresponding to the ligated nerves (L5 and L6) compared with the contralateral side, and compared with the ipsilateral side in the sham controls (Figure 4.14).

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**Figure 4.14:** (i) - (iii) Reference images of L4–L6 transverse sections from the Rat Brain Atlas (Paxinos and Watson, 1998). (iv) - (vi) Representative images of IB4-stained sections from regions L4–L6 of the spinal cord of sham control rats. (vii) - (ix) Representative images of IB4-stained sections from regions L4–L6 of the spinal cord of SNL rats. SNL-operated rats showed a qualitative decrease in IB4 staining intensity in laminae I-II of the dorsal horn of the spinal cord on the ipsilateral side in the regions corresponding to the sites of ligation (L5 and L6), compared with the contralateral side, and compared with the ipsilateral side of the sham controls. Scale bar = 130 µm.
4.3.3.3 Passive-avoidance testing

SNL and sham rats exhibited a passive-avoidance response following air-puff, but this response was not affected by SNL surgery: Friedman’s test showed an overall effect ($\chi^2_{(3)} = 12.536$, $p < 0.01$). Wilcoxon tests showed that air-puff produced a significant passive-avoidance response in both sham and SNL groups ($^*p < 0.05$), but there was no difference in responding between sham and SNL groups (Figure 4.15).

![Figure 4.15: SNL and sham rats exhibited a passive-avoidance response following air-puff but this response was not affected by SNL surgery. Both sham and SNL groups showed an increase in step-through latency in the retention trial compared with the acquisition trial but there was no difference in retention step-through latency between sham and SNL groups. Wilcoxon signed-ranks test: $^*p < 0.05$ acquisition vs. retention. Data are expressed as means ± SEM. $n = 5 – 6$.](image)

CFA and control rats exhibited a passive-avoidance response following air-puff, but this response was not affected by CFA treatment: Two-way repeated-measures ANOVA of log-transformed data revealed an overall effect of time ($F_{(1,26)} = 33.927$, $p < 0.001$). SNK post hoc tests showed that air-puff produced a significant passive-avoidance response in the CFA and control groups ($^{**}p < 0.01$, $^{***}p < 0.001$), but there was no difference in responding between the CFA and control groups (Figure 4.16).
Figure 4.16: CFA and control rats exhibited a passive-avoidance response following air-puff but this response was not affected by CFA injection. Both control and CFA groups showed an increase in step-through latency in the retention trial compared with the acquisition trial, but there was no difference in the retention step-through latency between control and CFA groups. SNK post hoc tests: **$p < 0.01$, ***$p < 0.001$, acquisition vs. retention. Data are expressed as means ± SEM. $n = 9$. 
4.4 Discussion

The aim of the present study was to investigate the cognitive domain of aversive learning and memory in models of chronic pain. As commonly used foot-shock induced passive-avoidance paradigms may be confounded by the use of noxious stimuli in this context, novel approaches for the measurement of aversive learning and memory were investigated. Two different stimuli were tested for their ability to induce a passive-avoidance response: ultrasound and air-puff. Under the present experimental conditions, ultrasound (21 kHz for 1 minute at a level of 100 ± 10 dB) was not found to induce a significant passive avoidance response. The reason for this is unclear, given the findings of Dokla et al. (1989). These authors observed a passive-avoidance response using ultrasound at a similar frequency and intensity. However, their experiments were conducted in Fischer rats as opposed to the Sprague-Dawley rats used in the present experiment, and strain differences in the behavioural and neurochemical responses to ultrasound have been demonstrated previously (Commissaris et al., 2000; Neophytou et al., 2000). For example, Lister-hooded rats exposed to ultrasound exhibited a startle response (characterised by brisk running and jumping) followed by a period of freezing behaviour, while Wistar rats had a prolonged period of freezing and did not show an initial escape response. c-Fos was expressed in both strains in the PAG following ultrasound exposure; however, expression was greater in the dorsal PAG of Lister-hooded rats and in the ventral PAG in Wistar rats (Commissaris et al., 2000; Neophytou et al., 2000). It is also important to note that the pre-acquisition habituation to the passive-avoidance arena used in later air-puff experiments was not included in the ultrasound paradigm. It is possible, therefore, that anxiety related to the introduction into a novel environment may have affected the test outcome. The present results do demonstrate that a single brief air-puff exposure was capable of producing a robust passive-avoidance response in rats in a simple context-conditioning paradigm and that this response is partially attenuated by pre-acquisition administration of the muscarinic receptor antagonist scopolamine,
at a dose of 3 mg/kg i.p. The passive-avoidance response was still expressed after 5 days of extinction training, suggesting that the air-puff-induced response was prolonged and robust. Further studies would be required to determine the time points at which the response is diminished or extinguished completely. The present findings also show that this validated air-puff paradigm successfully produces a passive-avoidance response in two different rat models of chronic pain: SNL, which models chronic neuropathic pain, and intraplantar injection of CFA, which models chronic inflammatory pain. Allodynia and hyperalgesia, confirmed behaviourally, indicated that SNL surgery and intraplantar CFA injection successfully resulted in neuropathic and inflammatory pain-like phenotypes, respectively. Furthermore, CFA resulted in hindpaw oedema and SNL surgery was associated with a qualitative decrease in IB4 at the L5 and L6 levels of the spinal cord. Although the lack of quantitative analysis of IB4 staining in this experiment represents a limitation, a marked decrease in the staining was qualitatively observed and the quantitation of IB4 in the SNL model was well characterised in the experiment described in Chapter 3. The level of IB4 staining was not assessed in CFA-treated rats. The reduction in IB4 staining in the SNL model is associated with damage to peripheral nerves and there is little evidence to suggest that peripheral inflammation is capable of damaging afferent nerve fibres. Although sensitivity and plasticity of IB4-positive nerves may be altered following hindpaw injection of CFA, a number of studies show that IB4 staining intensity is unaltered in the CFA model (Breese et al., 2005; Weng et al., 2012). Similarly, other markers of nerve damage such as ATF-3 (expressed in the dorsal root ganglion) are also unaffected in the this model (Inglis et al., 2005). Passive-avoidance responding was not altered in either pain model with respect to controls, suggesting that aversive learning and memory remained intact in both models.

As discussed in Chapter 1, there is a substantial body of evidence to support the idea that chronic pain is associated with cognitive impairment in human patients and in animal models of chronic pain, including both inflammatory and
neuropathic pain models. Deficits in attention and novel object recognition have been demonstrated in models of tonic persistent pain (formalin injection) and chronic (2,4,6-trinitrobenzene-induced colitis and CFA) inflammatory pain in rats (Boyette-Davis et al., 2008; Millecamps et al., 2004). Inflammatory pain, induced by intra-articular kaolin and carrageenan into the knee joint (Ji et al., 2010) or CFA injection into the hindpaw tibiotarsal joint (Pais-Vieira et al., 2009b) was also shown to affect the performance of rats in a gambling task which assessed emotional decision making. In these studies increased pain responding was associated with a preference for a “high-risk” lever associated with larger but more infrequent rewards than the alternative lever. Nerve-injured rats showed impaired cognition in the Morris water maze, in both spatial learning (Hu et al., 2010) and reversal tasks that evaluate cognitive flexibility (Leite-Almeida et al., 2009). There is substantial overlap in the neuroanatomical regions mediating aversive learning/memory and pain, including limbic regions such as the amygdala and hippocampus (Ambrogi Lorenzini et al., 1997; Apkarian et al., 2005; LeDoux, 1993; Lorenzini et al., 1996). Given this overlap, we thought it reasonable to hypothesise that aversive learning and memory, as cognitive constructs, may be particularly affected by chronic pain.

Suzuki et al. (2007) tested performance in a foot-shock-induced passive-avoidance paradigm in nerve-injured mice. The paradigm used a step-through light/dark arena and the aversive stimulus consisted of a 0.2 mA electric shock. These researchers reported that nerve injury was not associated with a deficit in aversive learning and memory under those conditions. However, as discussed earlier in this chapter, in circumstances where there is pre-existing hypersensitivity of the paws to noxious stimulation (as occurs in many animal models of chronic neuropathic and inflammatory pain), interpretation of a foot-shock-induced passive-avoidance response in the context of learning and memory is complicated and potentially confounded, as any change (or lack of change) in passive-avoidance responding could be a consequence of paw hypersensitivity to foot-shock rather than be-
ing due to alterations in cognitive processing. This theory is supported by the findings of Mutso et al. (2012). These authors reported that nerve-injured rats acquired a conditioned fear response to foot-shock, but did not successfully extinguish the response following repeated extinction trials. This difference may relate to alterations in the processing of foot-shock in nerve-injured rats. While no differences in the immediate behavioural response to foot-shock were detected in the study, central processing of the stimulus in the hippocampus was significantly altered in the neuropathic pain model, as shown by alterations in expression of ERK, a reduction in neurogenesis, and altered synaptic plasticity. The present study demonstrates that air-puff directed at the head/face can be used successfully as an alternative to foot-shock to produce a passive-avoidance response and avoid this potential confound, allowing for a more objective and ethically acceptable analysis of passive-avoidance responding in rodent models of chronic pain.

In our validation experiment, retention trial step-through latency in the air-puff saline group was 264.7 s (119.5–300 s) compared with 9.34 s (8.01–11.01 s) in the acquisition trial (data expressed as median and interquartile ranges). Notably, the magnitude of this avoidance response was similar to that observed in similar one-trial paradigms which used foot-shock to induce passive avoidance (Graham and Buccafusco, 2001; Holloway and Wansley, 1973). As with foot-shock (Elrod and Buccafusco, 1988), air-puff-induced passive avoidance was sensitive to the pre-acquisition administration of the muscarinic receptor antagonist scopolamine, indicating a learning-mediated phenomenon. Our results suggest that aversive learning and memory assessed in our air-puff passive-avoidance paradigm were not affected in either the inflammatory (CFA) or neuropathic (SNL) pain models under the conditions and at the time point described. This result supports the findings of (Suzuki et al., 2007) and other related research which has shown that learned helplessness in the forced-swim test and active avoidance in the place-escape/avoidance paradigm can be acquired in rat models of chronic pain (Gonçalves et al., 2008; Hu et al., 2009; Leite-Almeida et al., 2009; Suzuki et al.,
2007). These findings relating to aversive learning and memory are somewhat surprising given the evidence, discussed above, that chronic pain is associated with impairment in other cognitive domains, both clinically and preclinically. It is important to note that cognitive testing was performed at only one time point relative to spinal nerve ligation surgery/CFA injection. The mechanism underlying cognitive impairment in chronic pain has yet to be elucidated, but it has been hypothesised that pain-related alterations in brain structure and plasticity may in turn affect cognition (see Introduction). A study by Seminowicz et al. (2009) found anatomical alterations in brain regions associated with cognition following peripheral nerve injury in rats, but these alterations were not observed until several months post surgery. Therefore, although we observed robust, consistent expression of chronic pain-like behaviours at the post-surgery time point selected for passive-avoidance testing, it is possible that cognitive impairment, as assessed using this paradigm, may not have developed by this time point. However, the time point selected in the present study (days 18–20 post SNL or post CFA) was based on previous studies that have demonstrated pain-related alterations in both emotional (Suzuki et al., 2007) and cognitive (Pais-Vieira et al., 2009a) behaviours within a similar timeframe relative to injury. A recent study by Leite-Almeida et al. (2009) has indicated that age is another important factor in expression of pain-related cognitive impairment, with mid-aged (9-month-old) rats, but not young (3-month-old) or old (22-month-old) rats, showing an increased susceptibility to impaired performance in a Morris water maze reversal task. This impairment may be due to a variety of age-related alterations in neural plasticity, neurotransmitter and neuromodulator levels, structural changes in the brain, and neuroendocrine alterations. Although aversive learning and memory were not investigated by Leite-Almeida and colleagues, it is possible that pain-related deficits in these may also be detectable in mid-aged rats, despite failure to detect any deficits in the young adult rats (6–9 weeks old on arrival) used in the present study. However, it is also possible, given the results of the present
study and the findings of Suzuki et al. (2007), that aversive memory is simply unaffected in rodent models of chronic pain, or that passive-avoidance responding is not a reliable predictor of pain-related cognitive impairment. The learning and memory mechanisms affected by chronic pain may be distinct from those affected by scopolamine, and thus more specific behavioural paradigms may be required to detect cognitive deficits associated with chronic pain. Extinction of the air-puff induced passive-avoidance response in SNL or CFA models was not tested in the present study. In the light of the recent investigation by Mutso et al. (2012), in which impaired extinction of context-conditioned fear induced by foot-shock was observed in SNI rats, a detailed examination of the extinction profile in the novel air-puff paradigm is clearly necessary.

The use of air-puff to induce avoidance responding and to evaluate cognition has been described previously (Cimadevilla et al., 2001; Karlsson et al., 2009; Koistinaho et al., 2001). However, the apparatus and test protocols differ from those described in the present study. Karlsson et al. (2009) tested cognition in a multivariate concentric square-field paradigm, which was also used to investigate locomotor activity, exploration, risk assessment, risk-taking and security-seeking. Air-puff was administered when rats entered a defined zone within the test apparatus. The memory retention trial was carried out 2 weeks later and the percentage of rats returning to the air-puff-associated zone was recorded. The paradigm used by Cimadevilla et al. (2001) and Koistinaho et al. (2001) tested both active and passive avoidance in mice. The apparatus consisted of a circular arena, where entry into a segment of the arena was punished by an air-puff stimulus. In the passive-avoidance trial, mice spent less time in the punished segment of the arena, indicating memory of the aversive air-puff stimulus. In the active-avoidance test, the arena was rotated such that animals were required to avoid the air-puff-associated segment not only by not entering it, but also by not allowing themselves to be transported into it. This part of the test also involved a spatial component, as subjects had to learn the location of the punished segment according to extra-
maze cues. Passive and active avoidance were tested over a period of 4 days. The air-puff passive-avoidance paradigm described in the present study is relatively simple, and can be carried out over a shorter period of time, in comparison with those described above. Thus it lends itself to high-throughput cognitive screening of passive-avoidance behaviour. In addition, our paradigm is more similar to commonly used foot-shock paradigms, with the principal modification being the replacement of foot-shock with air-puff, allowing for a more direct comparison of results across paradigms.

A noteworthy feature of the passive-avoidance paradigm presented here is its ability to assess aversive learning and memory without directly activating peripheral nociceptive pathways and this may be of particular utility in certain circumstances. Clinical and preclinical studies have demonstrated that altered sensitivity to noxious stimuli, or increased incidence and severity of chronic pain, is associated with a large number of disorders of the central nervous system, including depression (Boettger et al., 2010; Dworkin et al., 1995; Klauenberg et al., 2008; Shi et al., 2010b,a; Terhaar et al., 2010), anxiety disorders (Asmundson and Katz, 2009; Finn et al., 2006; Geuze et al., 2007; Ploghaus et al., 2001; Rivat et al., 2010), multiple sclerosis (Kenner et al., 2007; Olechowski et al., 2009; Solaro et al., 2003) and others. Thus, a passive-avoidance paradigm using air-puff in place of noxious foot-shock may also be preferable for assessment of aversive learning and memory in rodent models of these disorders. In addition, many pharmacological agents (including, of course, analgesic drugs) may alter threshold to noxious stimuli. Therefore, passive-avoidance testing using an air-puff paradigm may yield a more accurate indication of the effect of such drugs on aversive learning and memory than testing using a foot-shock paradigm. Thus, the paradigm developed here may be useful in future studies aimed at assessing aversive learning and memory in rodent models of disease where pain processing or sensitivity to noxious stimuli is altered.

It should be noted that in the current study, response thresholds to mechani-
cal stimulation were lower post injury in both sham and needle-insertion control groups. Sham controls also had a greater number of responses to acetone application post surgery. These data suggest that the control groups also developed increased sensitivity to these mechanical and thermal stimuli. However, a similar increase in sensitivity was observed in Chapter 3 between the first test session and all subsequent test sessions and we believe it to be an artefact of the testing procedure used. It is also possible that the observed decreases in mechanical threshold and increases in response to cold stimulation may be due to experimenter bias. As behavioural pain testing requires subjective assessment of responses, the likelihood of the experimenter detecting a response post injury may increase. In spite of this limitation, there was a clear difference between both pain models and their respective control groups post injury in all of the behavioural pain tests, which would indicate that both SNL surgery and CFA treatment were associated with increased pain-related behaviours with respect to controls, and thus the potential to detect pain-related cognitive impairment should still have existed.

In conclusion, air-puff can be used to produce a reliable, robust passive-avoidance response in a context-conditioned aversive learning paradigm. Aversive learning and memory, as tested using the air-puff passive-avoidance paradigm, appear to be intact in models of chronic inflammatory and neuropathic pain, as step-through latency was not altered in SNL or CFA models compared with their respective controls.
Chapter 5  Characterisation of Cognitive Behaviour in the SNL Model in Mid-Aged Rats, and Synaptophysin Expression in the Hippocampus and PFC

5.1  Introduction

Chapters 3 and 4 investigated a number of different types of cognitive function in both neuropathic and inflammatory pain models. Neither the neuropathic SNL model nor the inflammatory CFA model was associated with any notable deficits in cognitive function. While these findings concur with some previous investigations (Suzuki et al., 2007), conflicting results have been reported in the majority of studies investigating the effects of pain on cognitive function (Boyette-Davis et al., 2008; Cain et al., 1997; Hu et al., 2010; Ji et al., 2010; Kodama et al., 2011; Lindner et al., 1999; Millecamps et al., 2004; Pais-Vieira et al., 2009a,b; Ren et al., 2011).

In 2009, Leite-Almeida et al. (2009) published the results of an experiment which highlighted the important influence of age on the interaction between pain and cognition. They found that a specific type of cognitive function, cognitive flexibility, was impaired in nerve-injured rodents but only in a mid-aged cohort of rats (as opposed to young or old rats). This finding is of particular interest given that age is associated with alterations in pain sensitivity, and that progressive cognitive decline is a function of increased age (for detailed discussion see Section 1.4). A number of clinical investigations have found pain-related deficits in cognitive function specifically in older age groups (Buckalew et al., 2010, 2008; Karp et al., 2006; Oosterman et al., 2009; Scherder et al., 2008; Weiner et al., 2006), while a study by Oosterman et al. (2011) directly examined the effect of
age on the interaction between pain and cognition, but found that age had no additive effect on memory impairment associated with pain. The type of cognitive function assessed by Leite-Almeida et al. (2009) is also of interest as the Morris water maze spatial reversal task involved is somewhat analogous to clinical tests of executive function such as attentional interference tasks, the Wisconsin Card Sorting Test and process dissociation tasks. Executive function has been shown to be negatively affected in chronic pain, using a variety of testing paradigms (Bosma and Kessels, 2002; Eccleston, 1994; Grisart and Plaghki, 1999; Grisart and Van der Linden, 2001; Karp et al., 2006; Ryan et al., 1993; Verdejo-García et al., 2009; Weiner et al., 2006). The processes of spatial learning, extinction and relearning involved in the reversal task possibly reflect an increased cognitive load compared with simple spatial learning and memory tasks, and it has been suggested that the interference effect of pain on cognitive function is greater, the more demanding the cognitive task (Eccleston, 1994). This may explain, in part, the lack of significant cognitive deficits observed in the two earlier experiments presented herein (Chapters 3 and 4).

The spared nerve injury (SNI) model of neuropathic pain (Decosterd and Woolf, 2000) used by Leite-Almeida et al. (2009) is a commonly used surgical technique that results in a neuropathic pain-like behavioural phenotype. It differs from the SNL model in that it involves ligation and axotomy of nerve fibres, and is performed on the tibial and peroneal branches of the peripheral sciatic nerve rather than on spinal nerves. No effects of SNI surgery on spatial learning in the traditional water maze task were found (Leite-Almeida et al., 2009), while L5 nerve transection was associated with impaired spatial learning and memory in a similar task (Hu et al., 2010). These findings suggest that the cognitive behavioural consequences, and their neural mechanisms, may differ depending on the pain model.

As discussed in Chapter 1, the precise mechanisms by which chronic pain may affect cognitive function are unclear. However, alterations in synaptic plasticity
play a key role in both pain and cognitive function. Synaptic connectivity has been shown to be altered in cognitive-associated brain regions such as the hippocampus (Kodama et al., 2007; Mutso et al., 2012; Ren et al., 2011; Zhao et al., 2009), the amygdala (Bird et al., 2005; Fu et al., 2008; Neugebauer et al., 2003) and the ACC (Zhuo, 2006, 2007) in models of chronic pain. Synaptophysin is a presynaptic protein which, because of its abundance and localization exclusively to synaptic vesicles, is commonly used as a marker of presynaptic terminals (Calhoun et al., 1998; Kwon and Chapman, 2011). The expression of synaptophysin has been shown to be altered in both age-related (Benice et al., 2006; Calhoun et al., 1998) and disease-related (King and Arendash, 2002; Seabrook et al., 1999) cognitive impairment, and synaptophysin knockdown in mice was associated with impaired learning and memory. Synaptophysin is altered in the periphery and spinal cord in animal models of chronic pain (Chou et al., 2002; Jaken et al., 2010; Lin et al., 2011; Peng et al., 2010; Sun et al., 2006) and recently pain-related cognitive impairment was shown to be associated with a decrease in the number of synaptophysin-positive boutons in the CA1 region of the hippocampus (Ren et al., 2011).

The goal of the present study was to expand on previous literature, in particular the findings of Leite-Almeida et al. (2009) and Ren et al. (2011). Specifically, the aims were:

- To investigate spatial reversal learning in mid-aged rats following SNL surgery to determine whether impaired reversal learning is conserved irrespective of the precise nature and location of the peripheral nerve injury.

- To examine the performance of mid-aged SNL rats in a battery of additional cognitive tests

- To quantify the expression of synaptophysin, as a marker of presynaptic terminals, in cognitive-associated brain regions, the medial prefrontal cortex (mPFC) and the CA1 region of the hippocampus.
CHAPTER 5. SNL MODEL IN MID-AGED RATS: COGNITION AND SYNAPTOPHYSGIN EXPRESSION

5.2 Methods

5.2.1 Animals

Mid-aged male Sprague-Dawley rats (Charles River, L’Arbresle, France) were used in this experiment. Rats were 9 months old on arrival at the facility and weighed 465–600g. As described in Chapter 2, experimental procedures were conducted with the approval of appropriate agencies and according to relevant ethical guidelines. Animals were housed under standard conditions of light and temperature as described in Section 2.2.1, and food and water were available ad libitum.

5.2.2 Experimental protocol

Rats were allowed to habituate to the facility for a period of 7 days, after which baseline sensory tests (von Frey, Hargreaves and acetone-drop tests, see Chapter 2, Section 2.2.3.2) were carried out over 3 days. The arena used for von Frey and acetone-drop tests in this experiment consisted of six adjoining chambers with dimensions 25 cm \((l)\times 20 \text{ cm } (w) \times 14 \text{ cm } (h)\). The sides were made of clear Perspex and the partitions between chambers were made from white melamine-coated chipboard. The Hargreaves arena was a three-chambered arena made from clear Perspex, with chamber dimensions 22 cm \((l)\times 20 \text{ cm } (w) \times 15 \text{ cm } (h)\). Rats were then randomly assigned to SNL \((n = 11)\) or sham \((n = 12)\) groups, and surgeries were performed as described in Chapter 2. Post-surgery von Frey testing was carried out on day 1 and every second day thereafter until day 13 to monitor the development of mechanical allodynia. Development of cold allodynia was assessed by acetone-drop test on days 4 and 12 post surgery, and thermal hyperalgesia by Hargreaves test on days 8 and 16. Wound debridement (under anaesthesia) was carried out on one animal from the SNL group to remove necrotic tissue and to promote healing of the surgical wound. The timing of this intervention was such that this animal could not be included in post-surgical Hargreaves testing and was therefore removed from the analyses of this test at all.
time points (i.e., final n numbers for the Hargreaves test were sham n = 12 and SNL n = 10).

Cognitive testing began on day 19 post surgery; full descriptions of all tests are provided in Chapter 2, Section 2.2.4. Rats were habituated to novel-object arena and recognition memory was assessed the following day. Three rats in the SNL group and one rat in the sham group were excluded from the novel object analyses as they failed to explore both objects during the familiarisation period (Exposure 1), and therefore would not have been able to distinguish between novel and familiar objects. Aversive memory was measured using the air-puff passive-avoidance paradigm on days 21–23 post surgery. Three rats from the SNL group and two rats from the sham control group did not enter the dark compartment of the arena within 300 seconds on the acquisition day. These rats were excluded from subsequent testing in this paradigm and were not included in the passive avoidance analyses (final n = 8–9). Morris water maze testing was carried out between days 46 and 60 post surgery. A one-day cued test was followed by 5 days of acquisition training and a one-day forward probe trial, rats then underwent 5 days of reversal training followed by a one-day reversal probe trial. The water maze used in this experiment was the larger of the two described in Chapter 2, having a diameter of 2 m. To confirm rats were still experiencing neuropathic pain-like symptoms, mechanical allodynia, thermal hyperalgesia and cold allodynia, they were reassessed on days 61–65 post surgery.

Ms. Claire O’Gorman MSc assisted in the acquisition and analysis of the behavioural data, specifically in relation to the manually scored behaviours in the novel object recognition task (sniffing, grooming, rearing, and attention directed at the objects/exploration of the objects).

At the end of the experimental period (day 65 or 67 post surgery), rats were sacrificed by transcardial perfusion with heparinised saline followed by 4% paraformaldehyde in phosphate buffer as described in Chapter 2. Brains were removed and stored in cryoprotectant (25% sucrose) solution until ready for sec-
tioning. Cryosectioning and immunohistochemistry were performed to determine the expression of the synaptic marker synaptophysin according to the methods described in Chapter 2. The study timeline is illustrated in Figure 5.1.

5.2.3 Statistical analysis

All data were tested for normality and homogeneity of variance, using Shapiro-Wilk and Levene’s tests, respectively. Parametric data were analysed using one-way or two-way ANOVA, or two-way repeated-measures ANOVA. Fisher’s least-significant-difference (LSD) tests or Student-Newman-Keuls (SNK) tests were used to make post hoc comparisons, as appropriate. Where possible, non-parametric data were log transformed and analysed similarly to parametric data. If non-parametric data could not be transformed, they were analysed using Friedman’s two-way ANOVA by ranks, followed by Mann-Whitney U or Wilcoxon signed-ranks tests. \( p \leq 0.05 \) was considered statistically significant. Data were analysed using SPSS software for Windows and results were depicted graphically with the aid of GraphPad Prism® software. For clarity of presentation, data are expressed as means ± SEM.

5.3 Results

5.3.1 Sensory testing

5.3.1.1 von Frey test

An overall Friedman’s ANOVA by ranks produced a significant result (\( \chi^2_{(17)} = 113.39, \ p < 0.001 \)). Friedman’s ANOVA by ranks on the individual groups revealed significant effects of time in both the sham (\( \chi^2_{(8)} = 36.06, \ p < 0.001 \)) and the SNL groups (\( \chi^2_{(8)} = 43.15, \ p < 0.001 \)). The 50% mechanical response threshold was decreased in the SNL group compared with the sham control group post surgery on days 3–13 and at the final time point day 62/64 post surgery, sug-
Figure 5.1: Experimental timeline

Day relative to surgery | Procedure
---|---
–12 | Animals housed
–4 to –1 | Baseline sensory testing
**Day 0** | **Surgery**
1 to 16 | Post-surgery sensory testing
19 and 20 | Novel object recognition
21 to 23 | Passive avoidance
46 and 48 | MWM Cued
47 to 54 | MWM Forward
53 to 60 | MWM Reversal
61 to 65 | Final sensory testing
**Day 65 or 67** | **Sacrifice**
Post-Mortem | Tissue Processing and Immunohistochemistry

Cognitive Testing
Figure 5.2: Effect of SNL surgery on the mechanical response threshold of the ipsilateral hindpaw. SNL rats expressed mechanical allodynia at all post-surgery time points compared with sham controls and with pre-surgery baseline. There was also a decrease in threshold in the sham controls post surgery compared with baseline. Mann-Whitney U tests: *p < 0.05, **p < 0.01, ***p < 0.001 SNL vs. sham; Wilcoxon signed-ranks test: ##p < 0.01 sham post surgery vs. sham baseline, ++p < 0.001 SNL post surgery vs. SNL baseline. Data are expressed as means ± SEM, n = 11 – 12.

suggesting persistent expression of mechanical allodynia (*p < 0.01, Mann-Whitney U tests). The response threshold was also significantly decreased post surgery in both sham (##p < 0.01, days 3–13 and 62/64) and SNL groups (++p < 0.01, days 1–13 and 62/64) compared with their respective baselines (Wilcoxon signed-ranks tests) (Figure 5.2).

5.3.1.2 Hargreaves test

The overall Friedman’s ANOVA by ranks was significant (χ²(7) = 37.10, p < 0.001). Friedman’s ANOVA by ranks for the SNL group indicated a significant effect of time in that group (χ²(3) = 16.56, p = 0.001). Mann-Whitney U tests showed
CHAPTER 5. SNL MODEL IN MID-AGED RATS: COGNITION AND SYNAPTOPHYSIN EXPRESSION

Figure 5.3: Effect of SNL surgery on ipsilateral hindpaw withdrawal latency in the Hargreaves test. Paw withdrawal latency was decreased in the ipsilateral hindpaw of SNL rats compared with sham rats on days 8, 16 and 63/65 post surgery (Mann-Whitney tests). Latency was also decreased in both sham (\#p < 0.05, days 8 and 16) and SNL group (\textdaggerdbl;p < 0.05, days 8, 16 and 63/65) compared with their respective baselines (Wilcoxon tests). Data are expressed as means ± SEM, n = 10 – 12.

that the paw withdrawal latency was decreased in the SNL group compared with the sham control group at all post-surgery time points (**p < 0.01, *p < 0.05).
Withdrawal latency decreased over time compared with baseline in the sham group on days 8 and 16 (\#p < 0.05) and in the SNL group on days 8, 16 and 63/65 (\textdaggerdbl;p < 0.01, Wilcoxon signed-ranks tests) (Figure 5.3). These results suggest expression of thermal hyperalgesia in the SNL group which was still evident 63–65 days post surgery.

5.3.1.3 Acetone-drop test

The overall Friedman’s ANOVA by ranks was significant ($\chi^2_{(7)} = 57.54$, $p < 0.001$). Friedman’s ANOVA by ranks also showed effects of time in both the sham ($\chi^2_{(3)} = 26.04$, $p < 0.001$) and the SNL ($\chi^2_{(3)} = 26.06$, $p < 0.001$) groups. The number of responses was significantly greater in the SNL group than in the control group
Figure 5.4: Effect of SNL surgery on the total number of hindpaw withdrawal responses following acetone application to the ipsilateral paw. The number of ipsilateral hindpaw withdrawal responses to acetone was significantly greater in SNL rats compared with sham controls on days 12 and 61/63 post surgery (Mann-Whitney U tests). A trend for a similar effect on day 4 was just below the level of statistical significance ($p = 0.056$, Mann-Whitney U test) There was an increase in the number of responses post surgery compared with baseline in both the sham ($\#p < 0.05$) and the SNL groups ($++p < 0.01$) compared with their respective baselines (Wilcoxon signed-ranks tests). Data are expressed as means ± SEM. $n = 11 - 12$. 

on days 12 and 61/63 post surgery, and there was a strong trend for a similar increase on day 4 ($p = 0.059$, Mann-Whitney U tests). The number of responses was also increased post surgery compared with baseline in both sham ($\#p < 0.05$) and SNL ($++p < 0.01$) groups compared with their respective baselines (Wilcoxon signed-ranks tests) (Figure 5.4). These data suggest persistent expression of cold allodynia in the nerve-ligated rats.

The percentage response to individual von Frey filaments along with the contralateral paw responses in the von Frey, Hargreaves and acetone-drop tests are presented graphically in Appendix B, Section B.2.
5.3.2 Cognitive testing

5.3.2.1 Novel object recognition

Discrimination ratios for the identical objects in Exposure 1, and familiar and novel objects in Exposure 2 were calculated according to the formula shown in Chapter 2, Section 2.2.4.1. Each exposure was analysed by one-way ANOVA, followed by post hoc tests (object was not considered as a main factor for the analysis as calculation of the discrimination ratio had already accounted for its effect, and one-way ANOVA is therefore appropriate). There were no differences in object exploration (object 1 vs. object 2) in either group or between groups in Exposure 1 (Figure 5.5). In Exposure 2, only the sham group displayed a significant preference for the novel object as shown by a greater discrimination ratio for the novel object than for the familiar object (**p < 0.01). Novel object exploration was significantly greater in the sham group than in the SNL group (#p < 0.05), suggesting there was a deficit in recognition memory in the nerve-injured rats (Figure 5.5). Duration of object exploration was analysed by two-way ANOVA with object and group as factors; there were no significant main effects (Figure 5.6). As in Chapter 3, the effect of nerve ligation on general behaviours was assessed in the 3-minute habituation period prior to object familiarisation. Frequency and duration of general behaviours (grooming and sniffing) were similar for both SNL- and sham-operated rats (Figures 5.8 and 5.9). SNL surgery was not associated with alterations in locomotor activity as measured by the total distance moved or by the velocity (horizontal activity, Figure 5.7). There was, however, an effect of SNL surgery on rearing behaviour, suggesting an impairment in vertical motor activity (Figure 5.8, p = 0.051). Despite this deficit, there was no difference between sham and SNL rats in the total time spent exploring the objects (which included the time spent rearing at the objects) in either exposure, indicating that the reduced novel object discrimination was not due to surgery-related impairment of movement.

As described in Chapter 2, the position of the novel object was counterbal-
Figure 5.5: Novel object recognition in sham and SNL rats. There were no differences in object discrimination in Exposure 1. In Exposure 2, sham rats only expressed a preference for the novel object. The increased exploration of the novel object was greater in the sham group than in the SNL group. Data are expressed as means ± SEM, n = 8 – 11. Fisher’s LSD post hoc analysis: **p < 0.01 sham novel vs. sham familiar; #p < 0.05 SNL novel vs. sham novel and SNL familiar vs. sham familiar.
Figure 5.6: Total duration of object exploration in novel object recognition. There were no differences in exploration between objects or groups in either exposure. Data are presented as means ± SEM, n = 9 – 11.

Figure 5.7: Locomotor activity of SNL and sham rats during the 3-minute novel object habituation trial. (a) Total distance moved. (b) Velocity. Data are expressed as means ± SEM, n = 8 – 11.
Figure 5.8: Duration of general and exploratory behaviours in SNL and sham rats during novel object habituation trial. There was a trend for a reduction in the duration of rearing the SNL group compared with the sham controls ($p = 0.051$, Student’s unpaired two-tailed $t$-test). Data are expressed as means ± SEM, $n = 8 – 11$.

Figure 5.9: Frequency of general and exploratory behaviours in SNL and sham rats during the 3-minute novel object habituation phase. Data are expressed as means ± SEM, $n = 8 – 11$. 
Figure 5.10: Apparent orientation bias in the novel object recognition test in SNL rats. There was no difference in the discrimination ratios for the objects on the left and right in the sham control group but the SNL group spent a greater proportion of time exploring the object on the left than the object on the right (SNK post hoc test: **p < 0.01, SNL left object vs. right object). Data are expressed as means ± SEM, n = 8 – 11.

anced to either the right or left side of the arena between rats to control for any orientation bias. The differences in novel object discrimination presented in Figure 5.5 and described above have accounted for this correction. Further analysis showed that in Exposure 2, when the nature of the object (novel or familiar) was ignored, the sham control group explored the objects on both sides of the arena equally, while the SNL group appeared to show a preference for the object on the left-hand side of the arena (Figure 5.10, **p < 0.01, SNK post hoc test), perhaps indicating a positional or orientational bias in the SNL group. Possible reasons for this are discussed in Section 5.4.

5.3.2.2 Air-puff passive avoidance

The air-puff passive-avoidance paradigm, described in detail in Chapter 4, was used to assess aversive memory in mid-aged SNL and sham control rats. An overall Friedman’s ANOVA including both groups and both time points was significant ($\chi^2_{(3)} = 18.52, p < 0.001$). The air-puff produced a significant passive avoidance response, as indicated by Wilcoxon signed-ranks tests, in both sham
and SNL groups (##p < 0.01, #p < 0.05 acquisition vs. retention) but there was no significant difference in the response between the sham and the SNL groups (Figure 5.11), suggesting that SNL surgery did not affect aversive memory as assessed in this paradigm.

Figure 5.11: Passive-avoidance response following air-puff in SNL and sham control rats. SNL and sham rats exhibited a passive-avoidance response following air-puff but this response was not affected by SNL surgery. Both the sham and SNL groups showed an increase in step-through latency in the retention trial compared with the acquisition trial but there was no difference in the retention step-through latency between sham and SNL groups. ##p < 0.01, #p < 0.05 acquisition vs. retention (Wilcoxon signed-ranks test). Data are expressed as means ± SEM, n = 8 – 10.

5.3.2.3 Morris water maze

Cued test

The latency to get onto the platform in the cued test (Figure 5.12) was analysed using Friedman’s ANOVA by ranks ($\chi^2_{(7)} = 17.19$, $p = 0.016$). There was a significant effect of time in the SNL group only ($\chi^2_{(3)} = 11.26$, $p = 0.010$). Latency
Figure 5.12: Latency to get onto the platform in mid-aged sham and SNL rats in the cued trial of the Morris water maze. The latency to get onto the platform decreased in the SNL group in trials 2 and 3 compared with trial 1. There was a trend for a decrease in the latency to get on to the platform in both groups in trial 4 (SNL \( p = 0.093 \), sham \( p = 0.086 \)). Data are expressed as means ± SEM, \( n = 11 \) – 12. Wilcoxon signed-ranks test: ++\( p < 0.01 \), +\( p < 0.05 \), SNL trials 2 and 3 vs. trial 1.

to the platform was decreased in trials 2 and 3 compared with trial 1 in the SNL group (++\( p < 0.01 \), +\( p < 0.05 \)). In trial 4, both sham and SNL groups showed a trend for a decrease in the latency to get onto the platform, but this did not reach the level of statistical significance (\( p = 0.093 \), SNL trial 4 vs. trial 1; \( p = 0.086 \), sham trial 4 vs. trial 1, Wilcoxon signed-ranks tests). There was no effect of SNL surgery at any time point. These results suggest that over the entire cued trial both sham and SNL rats, to some degree, learned to use the platform as an escape route from the maze.

**Acquisition training**

In the acquisition training phase, the path length to the platform (Figure 5.13) was analysed by a two-way repeated-measures ANOVA of log transformed data which revealed an overall effect of time (\( F_{(4,84)} = 21.97, p < 0.001 \)). There was no main effect of surgical treatment, or treatment by time interaction. Fisher’s LSD
Figure 5.13: Path length to locate the platform in sham and SNL rats in the acquisition phase of the Morris water maze. The path length decreased over days in both groups. Data are expressed as means ± SEM, n = 11 – 12. Fisher’s LSD post hoc tests: ##p < 0.01, sham days 2–5 vs. day 1; +++p < 0.001, SNL days 3–5 vs. day.

_post hoc_ tests showed that the path length decreased over time in both the sham (##p < 0.01, days 2–5 vs. day 1) and the SNL groups (+++p < 0.001, days 3–5 vs. day 1), which suggests rats successfully learned to locate the platform according to the extra-maze cues. There were no between-group differences, indicating that both groups learned equally well, in other words SNL surgery did not affect the acquisition of spatial reference memory. A similar pattern of results was observed for the latency to get onto the platform as for the path length and there was a significant decrease in swim speed over the 5-day acquisition period in both sham and SNL groups (see Appendix B, Section B.2).

**Forward probe trial**

The percentage distance moved in each of the quadrant zones, and the platform and annulus zone, by sham and SNL groups was compared by Student’s unpaired two-tailed _t_-tests. There were no differences in the percentage distance moved in any of the zones, suggesting there was no effect of SNL surgery on spatial memory.
Figure 5.14: % distance moved in arena zones during the probe trial in sham and SNL groups. Data are presented as means ± SEM, n = 11 – 12. The dashed line represents the % distance animals would move in each quadrant by chance (25%). Distance moved by both sham and SNL groups in the SW quadrant was above the level of chance.

as assessed by the paradigm. As expected the percentage distance moved in the SW quadrant, where the platform had been located, was above the level of chance (25%, see Figure 5.14). Results were similar when the percentage time spent in each zone was compared between treatment groups (see Appendix B, Section B.2).

Reversal training

The data for percentage distance moved in the SW quadrant (i.e, the old platform location) were analysed by a two-way repeated-measures ANOVA, which revealed a significant effect of time ($F_{(4,84)} = 20.43, p < 0.001$) and a significant interaction of time and surgery ($F_{(4,84)} = 2.76, p = 0.033$). Fisher’s LSD post hoc tests showed that the percentage distance moved in the SW quadrant decreased over time in both sham and SNL groups ($### p < 0.01$ sham days 2–5 vs. day 1, $+++ p < 0.01$ SNL days 3–5 vs. day 1). The proportion of distance moved in the SW quadrant,
i.e., the old platform location, was significantly greater in the SNL rats compared with the sham rats on day 2 (Figure 5.15). A similar result was observed in assessing the percentage time spent in the old quadrant location, with SNL rats spending a significantly greater proportion of time in the old quadrant location that the sham controls on day 2 of reversal training (Figure 5.16). These results suggest the SNL rats adapted poorly to the change in platform location compared with sham controls and tended to revert to the old location more frequently.

The percentage distance moved in the new platform location (NE quadrant) was assessed by a two-way repeated-measures ANOVA, which revealed a significant main effect of time ($F(4,84) = 16.92, p < 0.001$). There was no significant effect of surgery or surgery by time interaction. Fisher’s LSD post hoc tests showed that the percentage distance moved in the new platform location increased over time in both the sham and SNL groups ($##p < 0.01$ sham days 2–5 vs. day 1, $+p < 0.05$ SNL days 2–5 vs. day 1). There were no significant differences between sham and SNL groups at any of the time points (Figure 5.18). The percentage time spent in the NE quadrant was analysed by a two-way repeated-measures ANOVA (log-transformed data), which revealed a significant effect of time ($F(4,84) = 6.22, p < 0.001$) (Figure 5.19). Similar to the % distance moved in the NE quadrant there was an increase in the % time spent in the quadrant over time in the sham group ($#p < 0.05$, $##p < 0.01$, $###p < 0.001$). However, there was no increase over time in the SNL group and there was a trend for the sham group to spend a greater % of time in the new location than the sham controls on day 5 ($p = 0.071$, Fisher’s LSD post hoc test).

To investigate long-term adaption to the new platform location, the percentage distance moved and the percentage time spent in both the old location and the new location in the first trial only (of 4 trials each day) were also analysed (Figures 5.17 and 5.20). The data for percentage time spent in SW (old) quadrant were analysed by an overall Friedman’s ANOVA by ranks, which was significant ($\chi^2(9) = 29.93, p < 0.001$). Further Friedman’s tests within sham and SNL groups
Figure 5.15: % distance moved in the SW quadrant (old platform location) by sham and SNL rats (data averaged over 4 trials per day). Data are presented as means ± SEM, n = 11 – 12. The % distance moved in the old location decreased over time in both groups (Fisher’s LSD post hoc tests: ###p < 0.01 sham days 2–5 vs. day 1, +++p < 0.01 SNL days 3–5 vs. day 1). The % distance moved in the old quadrant was significantly greater in the SNL group than the sham control group on day 2 (*p < 0.05).

revealed an effect of time in the sham group only (χ^2_(4) = 17.41, p = 0.002). Wilcoxon signed-ranks tests showed that, in the sham group, the distance moved in the SW quadrant decreased compared with day 1 on days 2, 3 and 5 (#p < 0.01). There were trends for a decrease in the distance moved in the SW quadrant in the SNL group on days 4 and 5 (p = 0.062 and p = 0.075, respectively). The percentage distance moved by SNL rats in the SW quadrant was significantly greater than that of sham rats on day 2. A similar pattern of results for sham and SNL groups was observed for the percentage time spent in the SW quadrant in the first trial, though no significant time effects were observed (Appendix B, Section B.2).

The percentage distance moved in the new platform location (NE quadrant) in the first trial was analysed by Friedman’s ANOVA by ranks. The overall test was significant (χ^2_(9) = 36.14, p < 0.001) and there were significant effects of time in both sham (χ^2_(4) = 21.80, p < 0.001) and SNL groups (χ^2_(4) = 13.75, p = 0.008). Wilcoxon signed-ranks tests revealed an increase in the percentage distance moved
Figure 5.16: % time spent in the SW quadrant (old platform location) by sham and SNL rats (data averaged over 4 trials per day). Data are presented as means ± SEM, n = 11 – 12. The % time spent in the old location decreased over time in both groups (Fisher’s LSD post hoc tests: ##p < 0.01 sham days 2–5 vs. day 1, ++p < 0.01 SNL days 3–5 vs. day 1). The % time spent in the old quadrant was significantly greater in the SNL group than the sham control group on day 2.

Figure 5.17: % distance moved in the SW quadrant (old platform location) by sham and SNL rats (data for the first trial on each day). Data are presented as means ± SEM, n = 11 – 12. The % distance moved in the old location decreased over time in the sham group only (Wilcoxon signed-ranks tests: ##p < 0.01 sham vs. day 1). The % distance moved in the old quadrant was significantly greater in the SNL group than the sham control group on day 2.
in the NE quadrant over time in both sham (##p < 0.01, #p ≤ 0.05 sham days 2, 3 and 5 vs. day 1) and SNL groups (†p < 0.05 SNL days 3–5 vs. day 1). There were no significant differences in the distance moved in the NE quadrant between sham and SNL groups. The percentage time in the NE quadrant in the first trial was analysed similarly but there were no significant effects of time or surgical treatment (Appendix B, Section B.2). Similar to acquisition training there was a general decrease in the swim speed over days in the reversal training phase (see Appendix B).

Reversal probe trial
The percentage distance moved by sham and SNL rats in each of the defined zones was calculated (Figure 5.21). The percentage distance moved in the NE quadrant was, as expected, greater than the level of chance as this is where the platform had most recently been located. Student’s unpaired two-tailed t-tests were used
Figure 5.19: % time spent in the NE quadrant (new platform location) by sham and SNL rats (data averaged over 4 trials per day). Data are presented as means ± SEM, n = 11 – 12. The % time spent in the new location increased over time in the sham group only (two-way repeated-measures ANOVA (log-transformed data): #p < 0.05, ##p < 0.01, ###p < 0.001 sham vs. day 1). There were no differences between sham and SNL groups at any of the time points, but there was a trend for an increase in time spent in the NE quadrant in the sham group compared with the SNL group on day 5 (p = 0.071, Fisher’s LSD post hoc test).

Figure 5.20: % distance moved in the NE quadrant (new platform location) by sham and SNL rats (data for the first trial on each day). Data are presented as means ± SEM, n = 11 – 12. The % distance moved in the new location decreased over time in both sham and SNL groups (Wilcoxon signed-ranks tests: #p ≤ 0.05, ##p < 0.01 sham vs. day 1; †p < 0.05, SNL days 3–5 vs. day 1). There was no difference between sham and SNL groups at any time point.
CHAPTER 5. SNL MODEL IN MID-AGED RATS: COGNITION AND SYNAPTOPHYSIN EXPRESSION

Figure 5.21: % distance moved in arena zones during the reversal probe trial in sham and SNL groups. Data are presented as means ± SEM, n = 11 – 12. The dashed line represents the % distance animals would move in each quadrant by chance (25%). The % distance moved in the NE quadrant was above the level of chance for both sham and SNL groups. The sham rats distance moved in the NE quadrant as a % of their total distance moved during the trial was greater than that of the SNL rats (Student’s unpaired two-tailed t-test: *p ≤ 0.05).

Taken together, the results of the reversal training and reversal probe tasks suggest a pattern of impaired cognitive flexibility, which appeared to be particularly apparent when the intertrial intervals were at their greatest (comparing only the first trial on each day), with SNL rats tending to return to the old platform location more than sham controls. This may also be illustrated by tracking of the
Figure 5.22: Representative images of individual Ethovision® reversal tracks for an individual (a) sham or (b) SNL rat. Images indicate that the proportion of time and movement in the old location was greater in the SNL rats than in the sham controls.

paths of individual rats in the reversal training trials, which was done with the aid of Ethovision® software (see Figure 5.22).

5.3.3 Synaptophysin immunohistochemistry

Post mortem, immunohistochemistry was performed to determine the level of synaptophysin expression in discrete brain regions of a subset of sham and SNL rats that had undergone the battery of sensory and cognitive testing described above. The staining density, or area of positive synaptophysin staining as a percentage of the total tissue, was calculated. In the medial prefrontal cortex (mPFC), 6–8 images were gathered per section (3–4 per hemisphere), 6 sections per brain and 4–6 brains per group (sham and SNL). No significant hemispheric differences were observed and therefore images from the right and left sides of the brain were pooled. The total synaptophysin-positive percentage areas were averaged for sham and SNL groups and these groups were compared using Mann-Whitney U tests. There was a significant increase in the density of synaptophysin staining in the mPFC of SNL-operated rats compared with controls (***p < 0.001, Figure 5.23). Similar analyses were performed on images of the CA1 hippocampal
region (8 images per section, 6–7 sections per brain and 4–6 brains per group). Again, there was a significant increase in the synaptophysin staining density in SNL group compared with the sham control group (**p < 0.001, Figure 5.24). Figures 5.25 and 5.26 show representative images of the punctate synaptophysin staining observed in sham and SNL rats in both regions.

Figure 5.23: Density of synaptophysin staining in the mPFC of sham and SNL rats. There was a significant increase in the expression of synaptophysin in the mPFC of SNL-operated rats compared with sham controls (Mann-Whitney U test: ***p < 0.001). Data are presented as mean ± SEM. n = 4 – 6 brains per group, 6 sections per brain, and 6–8 images per section.

Figure 5.24: Density of synaptophysin staining in the CA1 region of the hippocampus of sham and SNL rats. There was a significant increase in the expression of synaptophysin in the CA1 of SNL-operated rats compared with sham controls (Mann-Whitney U test: ***p < 0.001). Data are presented as mean ± SEM. n = 4 – 6 brains per group, 6–7 sections per brain, and 8 images per section.
Figure 5.25: Representative images of synaptophysin immunostained sections of the mPFC. (a) $60\times$ image of mPFC of a sham control rat. (b) $60\times$ image of mPFC of SNL-operated rat. Density of synaptophysin was increased in the SNL group compared with the sham controls. (c) Diagram of the mPFC taken from the Rat Brain Atlas (Paxinos and Watson, 1998). The black squares represent the area from which the images were obtained.
Figure 5.26: Representative images of synaptophysin immunostained sections of the CA1 region of the hippocampus. (a) 60× image of the CA1 of a sham control rat. (b) 60× image of the CA1 of SNL-operated rat. Density of synaptophysin was increased in the SNL group compared with the sham controls. (c) Diagram of the CA1 taken from the Rat Brain Atlas (Paxinos and Watson, 1998). The black squares represent the area from which the images were obtained.
5.4 Discussion

SNL surgery in mid-aged rats was associated with mechanical and cold allodynia and thermal hyperalgesia. SNL-operated mid-aged rats showed impairments in the novel object recognition task and in the reversal task of the Morris water maze, but not in the air-puff passive-avoidance task. These results suggest that SNL surgery in mid-aged rats was associated with deficits in recognition memory and cognitive flexibility. These behavioural effects were accompanied by an increase in the expression of the synaptic vesicle protein synaptophysin in the CA1 region of the hippocampus and in the mPFC.

The present study demonstrates that SNL surgery can be used to model neuropathic pain-like behaviours in mid-aged Sprague-Dawley rats. It has been reported previously that behavioural signs of allodynia (mechanical and cold) are more pronounced in younger rats (5–7 weeks old) than in “mature” (3–5 months old) or old (15-month old) rats (Chung et al., 1995). Direct comparisons of neuropathic pain-like behaviour between the present study and those described in Chapters 3 and 4 are not strictly appropriate as the experiments were not matched in their design. However, a number of interesting differences in the surgery-induced phenotype were noted for animals of different ages across the studies. The mechanical response thresholds observed in the present study were similar to those in the first study (Chapter 3) which was performed in young animals, but higher than those observed in the SNL passive avoidance experiment (Chapter 4), also in younger rats. The study outlined in Chapter 3 was the first characterisation of the SNL surgery in this laboratory and, therefore, the higher response thresholds observed may have been a consequence of poor surgical or behavioural assessment techniques, due to inexperience. A comparison of the mechanical thresholds observed following SNL surgery in Chapter 4 and the present study suggest that, similar to the observation by Chung et al. (1995), behavioural signs of mechanical allodynia are more robust in younger rats. The present results also suggest, however, that differences in baseline mechanical sensitivity exist between different age cohorts,
a finding that was not noted by Chung et al. (1995). In contrast to the results of Chung et al. (1995), there were no apparent differences between the age groups in the response to cold stimuli following SNL surgery (comparing Chapter 4 and the present study), though it is important to note that different procedures were used to assess cold allodynia (Chung et al. used cold plate). Irrespective of age-related alterations in sensitivity, mechanical and cold allodynia and thermal hyperalgesia were evident in SNL mid-aged rats for up to 65 days post surgery compared with their mid-aged sham counterparts. Changes in sham sensitivity to mechanical, heat and cold stimuli were also observed in this experiment. As discussed in Chapter 3, we believe these changes to be an artefact of the test procedure, as opposed to an expression of neuropathic pain-like behaviour in the sham control group.

Novel object recognition was found to be impaired in mid-aged rats. The SNL group did not display a preference for the novel object compared with the familiar object and their exploration of the novel object was significantly lower than the sham control group. In a previous experiment (Chapter 3), a similar trend was observed but this did not reach the level of statistical significance. The increased magnitude of the pain-related effect in this experiment compared with that in Chapter 3, combined with the results of Leite-Almeida et al. (2009), may imply that mid-age represents the optimum age at which to detect pain-related impairment of cognitive function. Deficits in novel object recognition associated with pain have been demonstrated previously in younger rats in an inflammatory pain model (Millecamps et al., 2004) and mice in a neuropathic pain model (Kodama et al., 2011). The age-dependent effect observed here may, therefore, relate specifically to the rat SNL model of neuropathic pain. The side bias, observed specifically in SNL rats in the novel object recognition test, was an interesting finding of this study. A preference for the object on the left side of the arena was observed in SNL rats only, irrespective of whether the object was novel or familiar. It seems unlikely that this was a direct consequence of
the unilateral SNL surgery, as rats did not demonstrate spontaneous pain-related behaviours in the injured hindpaw, though it is possible that the reduction in rearing observed in this experiment in the SNL group compared with the sham group may reflect a unilateral impairment of vertical locomotor activity which may have affected the rats’ orientation. Another possibility is that the SNL surgery resulted in lateralised changes in neurotransmitter or neuromodulator level or lateralised alterations in synaptic plasticity. Such neural changes could affect object exploration in a unilateral manner, resulting in a form of neglect for one side of the arena (in this case the right/contralateral side). There were, however, no hemispheric differences in the expression of synaptophysin in SNL rats in the present study, and therefore such a hypothesis requires further investigation.

There were no effects of SNL surgery in mid-aged rats on performance in the air-puff passive avoidance paradigm. This, added to the results for younger SNL rats presented in Chapter 4 and the previous finding of Suzuki et al. (2007) appear to suggest that aversive memory is not affected in rodent models of neuropathic pain.

SNL surgery in mid-aged rats was not associated with any deficits in spatial learning or memory in the traditional water maze task. This is in agreement with the earlier findings in young SNL rats (Chapter 3). A study by Hu et al. (2010) found impairments in spatial learning and memory in the water maze in an L5 transection model of neuropathic pain. Thus, pain-related impairment of spatial memory may be dependent on the specific model of neuropathic pain used. The finding of impaired spatial reversal in the water maze in mid-aged rats supports and extends the work of Leite-Almeida and colleagues (2009), who showed a similar effect in mid-aged rats in a different model of neuropathic pain, the SNI model. The fact that this specific deficit was preserved across different models strengthens the case for a link between neuropathic pain and cognitive impairment. The observations also suggest that cognitive flexibility may be particularly sensitive to the effects of pain. Cognitive flexibility is analogous to executive functioning
in humans, and impaired executive functioning has been observed in chronic pain patients (Grisart and Van der Linden, 2001; Verdejo-Garcia et al., 2009).

In Chapter 1, it was hypothesised that pain-related changes in synaptic plasticity may be, in part, responsible for cognitive deficits in chronic pain. The number of synapses and the strength of synaptic connections is believed to be the cellular mechanism by which memory is encoded in the brain (Martin et al., 2000). Using fluorescence immunohistochemistry, the expression of the synaptic vesicle protein synaptophysin was found to be increased in mid-age SNL rats compared with sham controls in both the mPFC and the CA1 region of the hippocampus. The regions investigated were chosen on the basis of their relevance to the cognitive deficits observed in this study. Changes in synaptophysin expression have been shown previously in the hippocampus (Ren et al., 2011) and in the spinal cord in rodent models of neuropathic pain (Chou et al., 2002; Jaken et al., 2010; Lin et al., 2011; Peng et al., 2010); changes in other brain regions have not been investigated in these models.

The PFC, as discussed previously (Chapter 1), is involved in executive functions such as attention selection, response inhibition, task switching, planning and decision making. The PFC is also involved in working memory processes. The pre-limbic region of the medial PFC (mPFC) was examined in this study. Lesions of this subregion are associated with deficits in both delayed-non-matching-to-sample tasks (similar to the novel object recognition paradigm used in the present study) and reversal learning in the Morris water maze (Dalley et al., 2004; de Bruin et al., 1996). Structural and functional alterations in the prefrontal cortex have been shown previously both in pain patients and in animal models of neuropathic pain (Apkarian et al., 2004b; Luerding et al., 2008; Metz et al., 2009; Seminowicz et al., 2009), and dysfunction of amygdala-PFC circuitry has been purported as a mechanism for pain-related cognitive impairment. The expression of synaptophysin in the mPFC in relation to pain and cognition has not been investigated previously.
The hippocampus is known to be involved in learning and memory and spatial navigation. There has been some debate as to the involvement of the CA1 region of the hippocampus in novel object recognition (Mumby, 2001); however, it has been shown that the CA1 is important for working memory and spatial novelty (Steckler et al., 1998; Vago and Kesner, 2008) but not detection of novel objects. The novel object paradigm used in this study did not involve the use of intra- or extra-maze reference cues and, therefore, was likely to assess the recognition of both spatial and object novelty. Lesion of the CA1 region has also been shown to impair memory acquisition in the water maze (Stubley-Weatherly et al., 1996). The hippocampus is activated by the experience of pain (Apkarian et al., 2005) and there is some evidence for a decrease in total hippocampal volume in older adults with chronic pain (Zimmerman et al., 2009). Furthermore, pain is associated with alterations in hippocampal synaptic plasticity (Kodama et al., 2007; Mutso et al., 2012; Ren et al., 2011; Zhao et al., 2009), and recently a reduction in synaptophysin in the CA1 has been demonstrated in a model of pain-related cognitive impairment (Ren et al., 2011). Ren et al. (2011) showed that impairments in short-term and working memory were associated with a reduction in the number of synaptophysin-positive terminals in the CA1. Therefore, a reduction in the expression of synaptophysin in the CA1 (and possibly in the mPFC) was anticipated in this experiment. The opposing results observed here are surprising, though there are a number of possible explanations. The supraspinal representation of peripheral models of neuropathic pain has not been studied extensively, and synaptophysin protein expression in discrete brain regions may be different depending on the model used (SNI in the Ren et al. paper and SNL in the present study). The influence of age on the interaction between pain and cognition has been discussed at length, and the age discrepancy between the two studies may account for differences in synaptophysin levels in the CA1 and mPFC. These regions are implicated in normal age-related cognitive decline, and thus, the effects of pain may be expressed differently in the mid-aged rats used in the present study.

204
Despite these methodological differences between the studies, the behavioural outcomes of the study (impaired novel object recognition and impaired reversal) appear to imply an overall inhibition of hippocampal and prefrontal activity. The CA1 consists of layers or strata including the stratum pyramidale which is made up of primarily excitatory pyramidal cells. The stratum radiatum, imaged in the present study and in that of Ren et al. (2011), contains various types of interneurons, mossy fibres, basket cells and bipolar cells (Taupin, 2007). Hippocampal interneurons may be GABAergic, and synapse onto pyramidal cells resulting in inhibition (Cobb et al., 1997; Knowles and Schwartzkroin, 1981; Nunzi et al., 1985). Thus, an increase in synaptic terminal density could produce an overall inhibitory effect, corresponding to the cognitive behavioural deficits observed. It is possible that different neuronal subpopulations were imaged in the present study and in that of Ren et al. (2011) due to subtle regional variations in image acquisition. The presence of GABAergic interneurons in the mPFC (Retaux et al., 1993) allows for a similar mechanism to be hypothesised in this region. However, this explanation is speculative and requires further investigation. A double-labelling immunohistochemical study of synaptophysin, with markers of glutamatergic and GABAergic terminals to identify the nature of the terminals labelled by synaptophysin in both the CA1 and the mPFC would be informative in this regard.

In conclusion, the current experiment successfully replicated the finding of impaired reversal learning previously reported by Leite-Almeida et al. (2009), in a different surgical model of neuropathic pain. Moreover, this study demonstrated that other types of cognitive function are also disrupted in this model as the performance of mid-aged SNL rats in the novel object recognition task was also impaired. The expression of synaptophysin in cognitive-associated brain regions was increased, a finding that contradicts previous literature and warrants further investigation.
Chapter 6  Investigation of the Effects of Amitriptyline in the SNL Model in Mid-Aged Rats on Behaviour, Synaptophysin Expression and Monoamine Levels

6.1 Introduction

In Chapter 5, promising results were generated suggesting that pain-related deficits in cognition could be successfully modelled in both the novel object recognition task and in the Morris water maze reversal task, specifically in mid-aged rats. These deficits were found to be associated with alterations in synaptophysin expression in both the hippocampus and the prefrontal cortex. The present study aimed to investigate further the validity of this purported model, by attempting to replicate the findings of the previous study and the extent to which these deficits might be reversible with analgesic treatment. The neural mechanisms by which chronic pain may affect cognition were also examined further.

The tricyclic antidepressant (TCA) amitriptyline (Figure 6.1) is among the recommended first-line treatments for chronic neuropathic pain (Finnerup et al.,

![Figure 6.1: Chemical structure of amitriptyline.](image-url)
2005; O’Connor and Dworkin, 2009), based on a large number of randomised clinical trials and preclinical investigations that have demonstrated its effectiveness as an anti-neuropathic pain agent. TCAs are one of the older classes of antidepressant drugs but their use in the treatment of depression has declined in recent times due to the introduction of newer drugs with fewer adverse effects. Amitriptyline is a derivative of dibenzocycloheptadine and it is a tertiary amine tricyclic antidepressant (Bae et al. 2009). The efficacy of TCAs in depression may be explained in part by their ability to block the reuptake of noradrenaline and serotonin. However, the mechanism by which amitriptyline and related TCAs alleviate neuropathic pain has yet to be fully elucidated. In addition to their effects on noradrenaline and serotonin reuptake, TCAs can inhibit calcium reuptake, block sodium channels and activate potassium channels (Sindrup et al., 2005). TCAs also interact with opioid, adenosine histamine and acetylcholine signalling systems (Esser and Sawynok, 2000; Feighner, 1999; Valverde et al., 1994).

The cognitive effects associated with TCA treatment have been outlined in an earlier section (see Chapter 1, Table 1.4). The majority of the literature in this area appears to suggest either a negative effect of TCA treatment on cognitive function (Burgos et al., 2005; Naudon et al., 2007; Raja et al., 2002; Spring et al., 1992) or no effect (Podewils and Lyketsos, 2002). However, in a recent study by Hu et al. (2010), amitriptyline was found to improve cognitive function. This study involved a model of pain-related cognitive impairment induced by transection of the L5 spinal nerve which resulted in impaired performance in the Morris water maze learning and memory tasks. The deficit was reversed by chronic systemic administration of amitriptyline on days 7–28 post surgery. In the light of these findings, it may be hypothesised that amitriptyline has differential effects on cognition in the presence of chronic pain.

The role of the monoaminergic system in pain and cognition has also been discussed in Chapter 1. Though monoamine transmitters such as dopamine, noradrenaline and serotonin are known to be involved in pain (Kayser et al., 2007;
and cognition (Bunsey and Strupp, 1995; Scholes et al., 2007), few studies have directly investigated the involvement of the monoaminergic system in the interaction between pain and cognition. However, Pais-Vieira et al. (2009b) have shown that pain-related deficit in a rodent gambling task was associated with a decrease in levels of dopamine and its metabolites in the orbitofrontal cortex (Pais-Vieira et al., 2009b), and a reduction in dopamine and serotonin metabolites was also observed in the prefrontal cortex of rats following object exploration in a model of persistent inflammatory pain (Ford et al., 2008).

The primary goal of the present study was (1) to replicate the behavioural findings of the study described in Chapter 5 and to determine whether pain-related deficits in cognition, if present, could be reversed by chronic analgesic treatment with amitriptyline. Secondary aims were: (2) to investigate the role of monoamines in expression of neuropathic pain and pain-related cognitive impairment by measuring their concentrations in pain- and cognitive-associated brain regions and in the spinal cord, (3) to explore the mechanism of analgesic action of amitriptyline in neuropathic pain by determining whether its anti-neuropathic pain effect was associated with alterations in monoamine levels in discrete brain regions or in the spinal cord and (4) to expand on the finding of altered expression of synaptophysin (described in Chapter 5) by measuring protein expression using western immunoblotting.

### 6.2 Methods

#### 6.2.1 Animals

Mid-aged male Sprague-Dawley rats (Charles River, L’Arbresle, France) were used in this experiment. Rats were 9 months old on arrival at the facility and weighed 470–675 g. As described in Chapter 2, experimental procedures were conducted with the approval of the appropriate agencies and in accordance with the relevant
CHAPTER 6. SNL MODEL IN MID-AGED RATS: EFFECTS OF AMITRIPTYLINE

ethical guidelines. Animals were housed under standard conditions of light and temperature and food was available ad libitum. Animals were provided with 150 ml of normal tap water or amitriptyline dissolved in tap water, at a concentration of 0.3 g/l every 2 days. This volume was estimated as being in excess of normal consumption so animals were not considered to be water deprived.

6.2.2 Experimental protocol

The in vivo experimental protocol was similar to that described in Chapter 5, with some modifications. Rats were habituated to the facility for a period of 15 days, after which baseline sensory tests were carried out (see Chapter 2 for detailed description of methodologies). The arena used for von Frey and acetone-drop tests in this experiment consisted of six adjoining chambers with dimensions 25 cm (l) × 20 cm (w) × 14 cm (h). The sides were made of clear Perspex and the partitions between chambers were made from white melamine-coated chipboard. The Hargreaves arena was a six-chambered arena (chamber dimensions 11 cm (l) × 20 cm (w) × 15 cm, (h)) made from clear Perspex. Rats were then randomly assigned to one of four groups: sham-water, sham-amitriptyline (sham-ami), SNL-water or SNL-amitriptyline (SNL-ami). There were 9 animals per group in the SNL-water and sham-ami groups, 8 per group in the sham-water and SNL-ami groups. According to their treatment group, rats then underwent either sham or SNL surgery as described in Chapter 2. For rats receiving amitriptyline, the drug was dissolved in drinking water at a concentration of 0.3 g/l and provided at a volume of 150 ml in opaque water bottles on the day of surgery. This was replaced with 150 ml fresh water (with or without drug) every 2 days thereafter for the duration of the experiment. Sensory testing was carried out in the days immediately after surgery on a weekly basis over the entire duration of the experiment. The von Frey test was performed less frequently than the Hargreaves or acetone-drop tests as previous research has shown that mechanical allodynia is less sensitive to the anti-neuropathic effects of amitriptyline than other sensory
modalities (Bomholt et al., 2005; De Vry et al., 2004; Pradhan et al., 2010).

Cognitive testing consisted of the novel object recognition test, and the Morris water maze cued test, acquisition training, forward probe trial, reversal training and reversal probe trial, all carried out as described in detail previously in Chapter 2, Section 2.2.4. On day 70 post surgery, rats were sacrificed by decapitation and brains were removed and dissected as described in Chapter 2. Tissue from discrete brain regions and spinal cord was harvested, divided into left and right hemispheres, weighed, snap-frozen on dry ice and stored at $-80^\circ$C for subsequent processing. Monoamine levels were measured by high-performance liquid chromatography (HPLC) coupled to electrochemical detection. As fresh, rather than perfused, tissue was required for the monoaminergic assay, the expression of synaptophysin protein could not be measured by immunohistochemistry (as in Chapter 5), and was instead measured by western immunoblot. The western immunoblots for the amygdaloid and cerebral cortices were run by Ms. Azma Azeem MSc, but the analysis of all western immunoblot images was performed by the author of this thesis. Samples for all animals were analysed using both techniques, half being from the ipsilateral (left) hemisphere and half from the contralateral (right) hemisphere. Data from the right and left hemispheres were pooled where possible.

The experimental timeline is illustrated in Figure 6.2.

6.2.3 Statistical analysis

Behavioural data were analysed similar to previous studies (Chapters 2–5). All data were tested for normality and homogeneity of variance, using Shapiro-Wilk and Levene’s tests, respectively. Parametric data were analysed using one-way or two-way ANOVA, or two-way repeated-measures ANOVA. Fisher’s LSD tests were used to make post hoc comparisons, as appropriate. Where possible, non-parametric data were root transformed and analysed similarly to parametric data. If non-parametric data could not be transformed, they were analysed using Fried-
### Time relative to surgery

<table>
<thead>
<tr>
<th>Day Relative to Surgery</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day −20</td>
<td>Animals housed</td>
</tr>
<tr>
<td>Days −5 to −2</td>
<td>Baseline sensory testing vF, Hargreaves, Acetone</td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td>Surgery</td>
</tr>
<tr>
<td>Day 1 and 2</td>
<td>Hargreaves, Acetone</td>
</tr>
<tr>
<td>Week 1: days 7, 8 and 9</td>
<td>vF, Hargreaves, Acetone</td>
</tr>
<tr>
<td>Week 2: days 15 and 16</td>
<td>Hargreaves, Acetone</td>
</tr>
<tr>
<td>Week 3: days 21, 22 and 23</td>
<td>vF, Hargreaves, Acetone</td>
</tr>
<tr>
<td>Week 4: days 30 and 31</td>
<td>Hargreaves, Acetone</td>
</tr>
<tr>
<td>Days 35 and 36 or 37 and 38</td>
<td>Novel object recognition</td>
</tr>
<tr>
<td>Week 6: days 42, 43, 44</td>
<td>vF, Hargreaves, Acetone</td>
</tr>
<tr>
<td>Day 47</td>
<td>MWM Cued</td>
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<tr>
<td>Days 48 to 53</td>
<td>MWM Forward</td>
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<tr>
<td>Days 54 to 59</td>
<td>MWM Reverse</td>
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<tr>
<td>Week 9: days 63, 64 and 65</td>
<td>vF, Hargreaves, Acetone</td>
</tr>
</tbody>
</table>

### Day 70

<table>
<thead>
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<tbody>
<tr>
<td>Post-Mortem</td>
<td>Western blotting and HPLC</td>
</tr>
</tbody>
</table>

**Figure 6.2: Experimental timeline**
man’s two-way ANOVA by ranks, followed by Mann-Whitney $U$ or Wilcoxon signed-ranks tests. $p \leq 0.05$ was considered statistically significant.

Pooled left and right hemisphere data for western blot and HPLC were tested for normality and homogeneity of variance, using Shapiro-Wilk and Levene’s tests, respectively. Parametric (or approaching parametric) data were analysed using two-way ANOVA, with surgery and drug as factors. Fisher’s LSD tests were used to make post hoc comparisons, as appropriate. Non-parametric data were analysed by Kruskal-Wallis tests followed by Mann-Whitney $U$ tests where appropriate. Lateralised data were also tested for normality and homogeneity of variance, and parametric data were analysed by three-way ANOVA with surgery, drug and hemisphere as factors, followed by Fisher’s LSD post hoc tests as appropriate. Non-parametric data were analysed by an overall Friedman’s ANOVA by ranks including all groups and both hemispheres, followed by within-hemisphere Kruskal-Wallis tests and Mann-Whitney comparisons as appropriate.

Data were analysed using SPSS software for Windows and results were depicted graphically with the aid of GraphPad Prism® software. For clarity of presentation, data are expressed as means $\pm$ SEM.

### 6.3 Results

#### 6.3.1 Amitriptyline dosing

Fluid consumption and body weight were monitored in all four experimental groups every 1–2 days, and the dose of amitriptyline in the sham-ami and SNL-ami groups was calculated. The average results for the entire experimental period are presented in Table 6.1, and dose information and body weight averaged per week are presented in Figures 6.3 and 6.4. Fluid consumption was analysed by two-way ANOVA with surgery (SNL or sham) and drug (amitriptyline or water) as factors. There was a significant main effect of drug ($F_{(3,272)} = 79.569$, $p < 0.001$), and Fisher’s post hoc analysis showed that the average fluid consumption over the ex-
perimental period was significantly lower in the amitriptyline groups than in the groups receiving water (***$p < 0.001$ sham-ami vs. sham-water, +++$p < 0.001$ SNL-ami vs. SNL-water). There was no difference in the fluid consumption between the sham-ami and SNL-ami groups. There was, however, a statistically significant difference in the dose consumed by the two groups, with the SNL-ami group consuming slightly more of the drug on average (unpaired two-tailed $t$-test: ###$p < 0.001$, sham-ami vs. SNL-ami). This was also evident in the graph of weekly dose consumption. The weekly dose data were analysed by Friedman’s ANOVA by ranks, which revealed a significant effect overall ($\chi^2_{(19)} = 111.69$, $p < 0.001$), and significant effects of time in both sham-ami ($\chi^2_{(9)} = 56.86$, $p < 0.001$) and SNL-ami ($\chi^2_{(9)} = 25.88$, $p = 0.002$) groups. Dose was lower in the SNL-ami group than in the sham-ami group in week 3 and weeks 5–10 (**$p < 0.01$, *$p < 0.05$, Mann-Whitney $U$ tests) (Figure 6.3).

Body weights were analysed by Friedman’s ANOVA by ranks, revealing a significant effect ($\chi^2_{(4)} = 187.331$, $p < 0.001$). Mann-Whitney $U$ tests then showed that the average body weight was lower in the SNL-water and sham-ami groups than in the sham-water group (**$p < 0.001$), and in the SNL-ami group compared with the sham-ami group (###$p < 0.001$). The combination of surgery and amitriptyline treatment reduced average body weight relative to the SNL-water group (+++$p < 0.001$). Again, this was evident when body weight was analysed across weeks. Friedman’s ANOVA by ranks revealed a significant effect overall ($\chi^2_{(49)} = 914.21$, $p < 0.001$) and effects of time in all four treatment groups ($p < 0.001$). Kruskal-Wallis tests revealed significant effects of group at time points ($p < 0.001$). Both SNL surgery and amitriptyline treatment were associated with decreases in body weight per se, and the combination of SNL and amitriptyline had a marked effect on body weight (Figure 6.4).
Table 6.1: Fluid consumption, body weight and amitriptyline dosing. Average body weight was lower in the SNL-water and sham-ami groups than in the sham-water group (**p < 0.001). Average body weight was also lower in the SNL-ami group relative to the SNL-water group (+++p < 0.001) and compared with the sham-ami group (###p < 0.001). Rats receiving amitriptyline drank less fluid on average than rats receiving water (**p < 0.001, +++p < 0.001 vs. respective water control group). The dose of amitriptyline was significantly higher in the SNL-ami group than in the sham-ami group (###p < 0.001).

Figure 6.3: Amitriptyline dosing in sham and SNL rats expressed per week post surgery. The dose of amitriptyline consumed was lower in SNL rats than in sham controls at discrete time points over the course of the study (**p < 0.001, *p < 0.01, *p < 0.05, sham vs. SNL, Fisher’s LSD post hoc tests). Individual points are average doses for 8–9 rats over 6–7 days; data presented as means ± SEM.
Figure 6.4: Body weight of rats expressed per week post surgery. Body weight was affected both by SNL surgery ($p < 0.05$, SNL-water vs. sham-water; $p < 0.05$, SNL-ami vs. sham-ami) and by administration of amitriptyline ($p < 0.05$, sham-ami vs. sham-water; $p < 0.05$, SNL-ami vs. SNL-water). Rats that underwent the combination of SNL surgery and amitriptyline treatment had notably decreased body weights compared with all other treatment groups. Individual points are average weights for 8–9 rats over 6–7 days; data presented as means ± SEM.
6.3.2 Sensory testing

6.3.2.1 von Frey test

An overall Friedman’s ANOVA by ranks showed a significant result ($\chi^2_{(19)} = 98.95$, $p < 0.001$), and significant effects of time were also shown by Friedman’s tests in all four groups ($p < 0.01$). Kruskal-Wallis tests revealed significant effects of group in weeks 3, 6 and 9 ($p < 0.01$). Post hoc Mann-Whitney U tests showed that the response threshold was lower post surgery in both SNL-water and SNL-ami groups compared with their respective sham controls (##$p < 0.01$, sham-water vs. SNL water weeks 3–9; *$p < 0.05$, sham-ami vs. SNL-ami weeks 3–9). The response threshold was also significantly lower in the SNL-ami group than the sham-ami group at baseline (##$p < 0.01$), and there was a reduction in the sham-ami group compared with the sham-water group at week 1 post surgery (##$p < 0.05$). There was a decrease in response threshold post surgery compared with baseline in all four treatment groups (+$p < 0.05$) (Figure 6.5). These results indicate expression of mechanical allodynia in the SNL-operated rats which was not reversed by chronic oral treatment with amitriptyline. However, as basal sensitivity appeared to be higher (i.e., lower threshold) in the SNL-ami group, the ability to detect effects in this group post surgery may have been confounded. At week 1 post surgery, the sham-ami group had a lower threshold than the sham-water group, possibly indicating drug-associated hypersensitivity at this time point.

6.3.2.2 Hargreaves test

An overall Friedman’s ANOVA by ranks test produced a significant result ($\chi^2_{(31)} = 118.92$, $p < 0.001$), and Friedman’s tests also showed significant effects of time in the SNL-water ($\chi^2_{(7)} = 31.328$, $p < 0.001$) and the SNL-ami ($\chi^2_{(7)} = 22.917$, $p = 0.002$) groups. Kruskal-Wallis tests revealed main effects of group at weeks 2–9 post surgery ($p < 0.05$), and significant between-group differences were identi-
Figure 6.5: Effect of treatment on the mechanical response threshold of the ipsilateral hindpaw. SNL rats, irrespective of amitriptyline treatment, expressed mechanical allodynia at all post-surgery time points compared with sham controls (Mann-Whitney U tests: ##p < 0.01, SNL-water vs. sham-water; *p < 0.05, SNL-ami vs. sham-ami) and with pre-surgery baseline. There was also a decrease in threshold in the sham controls post surgery compared with baseline (Wilcoxon signed-ranks tests: +p < 0.05, all treatment groups weeks 1-9 post surgery vs. baseline). Amitriptyline treatment was associated with a lower withdrawal threshold in the sham group at week one post surgery (Mann-Whitney U test: Ωp < 0.05, sham-ami vs. sham-water), but had no other modulatory effects on allodynia associated with SNL surgery or effects in its own right. The SNL-ami group had a significantly lower response threshold at baseline than the SNL-water group (Mann-Whitney U test: **p < 0.01). Data are shown as means ± SEM. n = 8 – 9.
fied using Mann-Whitney $U$ tests. The hindpaw withdrawal latency was significantly lower in the SNL-water group than in the sham-water group ($###p < 0.001$, $#p < 0.05$), and in SNL-ami group compared with the sham-ami group ($*p < 0.05$), indicating expression of thermal hyperalgesia. However, the response latency was also significantly increased in the SNL-ami group compared with the SNL-water group ($^+p < 0.05$, weeks 3–9), which suggests that amitriptyline partially reversed the decrease in withdrawal latency associated with SNL surgery (Figure 6.6). Wilcoxon signed-ranks tests showed that the response latency decreased post surgery compared with baseline in both SNL groups (SNL-water, weeks 2–9 vs. baseline; SNL-ami, day 1 and weeks 1–9 vs. baseline) but not in the sham groups.

### 6.3.2.3 Acetone-drop test

An overall Friedman’s ANOVA showed a significant result ($\chi^2_{(31)} = 160.60$, $p < 0.001$), and further investigation showed significant effects of time in all four treatment groups ($p < 0.01$). Kruskal-Wallis tests also revealed an effect of group at weeks 3, 6 and 9 post surgery ($p < 0.05$), and the effects were approaching the level of significance at weeks 2 and 4 ($p = 0.066$ and $p = 0.078$, respectively). Mann-Whitney $U$ tests showed that there was a significantly greater number of responses in both SNL groups compared with their respective sham control groups ($###p < 0.001$, $##p < 0.01$, SNL-water vs. sham water, ###$p < 0.001$, **$p < 0.01$, *$p < 0.05$, SNL-ami vs. sham-ami) at all post-surgery time points. There was a slight trend for amitriptyline to partially reverse the SNL-associated increase in the number of responses at week 3, but this did not reach statistical significance ($p = 0.099$, SNL-ami vs. SNL-water). Wilcoxon signed-ranks tests revealed an increase in the number of responses post surgery compared with baseline in all four treatment groups ($^+p < 0.05$) (Figure 6.7). These results indicate that SNL surgery was associated with expression of cold allodynia but that this effect was not sensitive to treatment with amitriptyline.
Figure 6.6: Effect of treatment on ipsilateral hindpaw withdrawal latency in the Hargreaves test. Paw withdrawal latency was lower in the SNL-ami group than in the sham-ami group (*p < 0.05), and lower in the SNL-water group than in the sham-water group (#p < 0.05, ###p < 0.001, Mann-Whitney U tests) post surgery. The latency was increased in the SNL-ami group compared with the SNL-water group (+p < 0.05, Mann-Whitney U tests), suggesting a partial attenuation of the hyperalgesic response. Latency was also decreased in both the SNL-ami (day 1 and weeks 1–9) and SNL-water groups (weeks 2–9) compared with their respective baselines (Wilcoxon tests). Data are shown as means ± SEM. n = 8–9.
Figure 6.7: Effect of treatment on the total number of hindpaw withdrawal responses following acetone application to the ipsilateral paw. The number of ipsilateral hindpaw withdrawal responses to acetone was significantly greater in SNL-water and SNL-ami groups than in their comparative sham groups (Mann-Whitney $U$ tests: $##p < 0.01$, $###p < 0.001$, sham-water vs. SNL-water; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, SNL-ami vs. sham-ami). There was an increase in the number of responses post surgery compared with baseline in all four treatment groups ($^+p < 0.05$, Wilcoxon signed-ranks tests) compared with their respective baselines. Data are shown as means ± SEM. $n = 8−9$. 
The percentage response to individual von Frey filaments along with the contralateral paw responses in the von Frey, Hargreaves and acetone-drop tests are presented graphically in Appendix B, Section B.3.

6.3.3 Cognitive testing

6.3.3.1 Novel object recognition

Discrimination ratios were calculated according to the formula shown in Chapter 2 and analysed by a one-way ANOVA followed by post hoc tests. There were no differences in the exploration of object 1 and object 2 in Exposure 1 in any of the four treatment groups. In Exposure 2, rats displayed a preference for the novel object compared with the familiar object irrespective of their treatment (Figure 6.8). There were no between-group differences in the level of novel object discrimination. These data suggest that recognition memory was intact in all four treatment groups and that there were no effects of SNL surgery or amitriptyline. In Exposure 2, there was a significant main effect of object on the duration of object exploration (two-way ANOVA: $F_{(1,58)} = 8.344, p = 0.005$); the only significant pairwise comparison was an increase in the exploration time of the novel object compared with the familiar one in the sham-ami group (*$p < 0.05$) (Figure 6.9).

Similar to the previous experiments presented herein (Chapter 3 and Chapter 5), there were no significant effects of SNL surgery on locomotor activity (distance moved and velocity; Figure 6.10) or on general behaviours of sniffing, grooming, rearing and total exploration (sum duration of sniffing and rearing) (Figure 6.11). Amitriptyline was also shown to have no significant effects on locomotor or general behaviours. Based on results presented in Chapter 5, we also investigated whether there were any apparent orientational biases in object exploration. SNL surgery, in the absence of amitriptyline treatment, was associated with a preference for the object on the right-hand side of the arena compared with that on the left in Exposure 2 (*$p < 0.05$, Fisher’s LSD post hoc test), a preference that was not observed in SNL rats treated with amitriptyline (Figure 6.12).
CHAPTER 6. SNL MODEL IN MID-AGED RATS: EFFECTS OF AMITRIPTYLINE

Figure 6.8: Effect of treatment on novel object recognition in sham and SNL rats. There were no differences in object discrimination in Exposure 1. In Exposure 2, all four treatment groups expressed a preference for the novel object (**p < 0.01 sham novel vs. familiar). Data are shown as means ± SEM. n = 6 – 9.

Figure 6.9: Total duration of object exploration in novel object recognition. There were no differences in exploration between objects or groups in Exposure 1. In Exposure 2, the sham-ami group spent significantly longer exploring the novel object than the familiar one (*p < 0.05, novel object vs. familiar object, Fisher’s LSD post hoc test). There were no between-group differences in Exposure 2. Data are presented as means ± SEM, n = 7 – 9.
Figure 6.10: Locomotor activity during the novel object habituation phase. (a) Total distance moved. (b) Velocity. There were no significant differences between any of the treatment groups in either distance moved or velocity. Data are shown as means ± SEM. $n = 6 - 11$.

Figure 6.11: Duration of general and exploratory behaviours during novel object habituation phase. There were no significant differences between any of the treatment groups in general or exploratory behaviours. Data are expressed as means ± SEM. $n = 6 - 9$. 
Figure 6.12: Apparent orientation bias in the novel object recognition test in SNL-water rats. There was no difference in the discrimination ratios for the objects on the left and right in the sham-water, sham-ami or SNL-ami groups. The SNL-water group spent a greater proportion of time exploring the object on the right than the object on the left (Fisher’s LSD post hoc test: *$p < 0.05$, SNL-water left object vs. right object). Data are expressed as means ± SEM. $n = 6 – 9$.

6.3.3.2 Morris water maze

Cued test

A Friedman’s ANOVA by ranks including all groups and time points was found to be significant ($\chi^2_{(15)} = 58.73$, $p < 0.001$); effects of time were shown by Friedman’s tests in sham-water, sham-ami and SNL-water groups ($p < 0.01$), and the effect was approaching significance in the SNL-ami group ($p = 0.066$) (Figure 6.13). There were no significant effects of group in any of the trials (Kruskal-Wallis tests). Wilcoxon signed-ranks tests showed that the latency to get on to the platform decreased significantly over trials, compared with trial 1, in all four groups ($^*p < 0.05$, sham-ami, sham-water and SNL-water, trials 2–4 vs. trial 1; $^#p < 0.05$, SNL-water, trials 2–3 vs. trial 1). In trial 3, the sham-ami group took significantly longer to find the hidden platform than sham-water controls. These results imply an intact ability to identify the platform as the escape route from the maze in all treatment groups, and that the treatments were not associated with visual or motor impairments. The reason for the amitriptyline-associated increase on day 3 ($^*p < 0.05$, sham-ami vs. sham-water) was unclear.
Figure 6.13: Latency to get onto the platform in the cued trial of the Morris water maze. The latency to get onto the platform decreased in the sham-water, sham-ami and SNL-water groups in trials 2–4 compared with trial 1 ($^+$p < 0.05), and in the SNL-ami group in trials 2 and 3 compared with trial 1 ($#$p < 0.05) (Wilcoxon signed-ranks tests). The reason for the latency increase in the sham-ami group on day 3 (*p < 0.05, sham-ami vs. sham-water) is unclear. Data are expressed as means ± SEM. n = 8 – 9.

Acquisition training

The overall Friedman’s ANOVA for acquisition path length produced a significant result ($\chi^2_{(19)} = 54.42$, p < 0.001), and further tests revealed effects of time in all groups (p < 0.05), with the exception of the SNL-water group. Kruskal-Wallis tests revealed no significant effects of group on any of the acquisition days. Wilcoxon signed-ranks tests showed that the path length to the platform decreased in the sham-ami group on days 2–5 ($^+$p < 0.05) and in the sham-water group on days 3–5 ($#$p < 0.05) compared with day 1 (Figure 6.14). However, path length in the SNL-ami group decreased only on day 4 ($^*$p < 0.05) compared with day 1, and in the SNL-water group a decrease was only apparent at day 5 ($^{##}$p < 0.01). These results indicate that spatial learning may have been impaired or delayed in the SNL-operated rats; however, no significant between-group differences were observed. Similar results were obtained when latency to get onto the platform was analysed (Appendix B, Section B.3). There were also significant drug effects
Figure 6.14: Path length to locate the platform in the acquisition phase of the Morris water maze. The path length decreased over days all four treatment groups. The path length was decreased significantly on days 2–5 vs. day 1 in the sham-ami group (+p < 0.05), on days 3–5 in the sham-water group (#p < 0.05), on day 4 in the SNL-ami group (*p < 0.05) and on day 5 in the SNL-water (φφp < 0.01) (Wilcoxon signed-ranks tests). Data are expressed as means ± SEM. n = 8 – 9.

on swim speed during acquisition, with sham-ami rats having a faster swim speed than the sham-water group and either SNL group (Appendix B, Section B.3).

Forward probe
The percentage distance moved in the SW quadrant was above the level of chance in all four treatment groups (Figure 6.15), which suggests that irrespective of treatment, rats learned the position of the platform and were successfully able to recall the position in the probe trial. Percentage distance moved in each of the four quadrants and the zone containing the platform and the annulus were analysed by two-way ANOVA with surgery and drug as factors. In the case of the quadrant of interest, the SW quadrant (where the platform had been located), the surgery × drug interaction effect was approaching significance (F(1,30) = 2.97, p = 0.095) and there was a trend for the percentage distance moved in the SW quadrant to be lower in the sham-ami group than the sham water group, suggesting possible impaired memory for the platform location in this group. Interestingly, the amitriptyline effect was not observed in the SNL-ami group, suggesting differential
effects of amitriptyline in the presence or absence of pain. There were no other between-group differences in the percentage distance moved in the SW quadrant or in the platform and annulus zone.

Reversal training
The percentage distance moved in the SW quadrant (old platform location) (Figure 6.16) and the NE quadrant (new platform location) (Figure 6.17) were analysed using non-parametric statistics. The overall Friedman’s ANOVA was not significant in either case. For the percentage distance moved in the SW quadrant, no effects of time or group were indicated by Friedman’s or Kruskal-Wallis tests. A Friedman’s ANOVA by ranks revealed an effect of time in the percentage distance moved in the NE quadrant in the sham-ami group only ($\chi^2(4) = 12.53$, $p = 0.014$). In this group, the percentage distance moved in the NE quadrant was increased
Figure 6.16: % distance moved in the SW quadrant (old platform location). There were no changes in the % distance moved in the SW quadrant over time and there were no between-group differences at any of the time points. Data are presented as means ± SEM, \( n = 8 – 9 \).

on days 4 and 5 of reversal training compared with day 1 (\( p < 0.05 \), Wilcoxon signed-ranks tests). Similar results were obtained for the percentage time spent in the SW and NE quadrants and the corresponding figures are presented in Appendix B, Section B.3.

As in Chapter 5, the percentage distance moved and the percentage time spent in the old location and the new location during the first trial each day were also analysed to investigate long-term adaption, but no significant differences were observed.

Reversal probe trial
The percentage distances moved in each of the individual quadrant zones, and in the zone of the platform and annulus, were analysed by two-way ANOVAs with surgery and drug as factors. No main effects of either factor were observed in the quadrants of interest (SW, NW or platform + annulus). A similar result was obtained for the percentage time in each zone (Appendix B, Section B.3).
Figure 6.17: % distance moved in the NE quadrant (new platform location). The % distance moved in the new location increased over time in the SNL-ami group only (Wilcoxon signed-ranks tests: \(+p < 0.05\), days 4 and 5 vs. day 1). No other time- or group-related differences were observed. Data are presented as means ± SEM, \(n = 8–9\).

The percentage distance moved in the NW quadrant (new platform location) was below the level of chance in the sham-ami group suggesting poor retention of the new location in this group. For the other treatment groups, the percentage distance moved in the NE quadrant was greater than 25% but still relatively low compared with the previous experiment (see Chapter 5). Furthermore the average percentage distance moved in the SW quadrant (old location) was also above the level of chance in all treatment groups, with the exception of the sham-ami group (Figure 6.18). This indicates that rats failed to extinguish the memory of the old location and did not successfully adapt to the new location.

The results of the reversal training and reversal probe trial indicate that reversal learning and cognitive flexibility were impaired in all of the treatment groups including the sham-water control group. Thus, it is not possible to draw conclusions regarding the effects of SNL surgery or amitriptyline administration on these cognitive outcomes.
Figure 6.18: % distance moved in arena zones during the reversal probe trial in sham and SNL groups. Data are presented as means ± SEM, n = 8 – 9. The dashed line represents the % distance animals would move in each quadrant by chance (25%). There were no significant differences between treatment groups in the % distance moved in any of the zones of interest (SW and NE quadrants and platform + annulus).

6.3.4 Post-mortem analysis

Despite the lack of a behavioural phenotype of cognitive impairment in this experiment, SNL surgery was nonetheless associated with characteristic neuropathic pain-like behaviours, some of which were reversed by administration of amitriptyline. Therefore, post-mortem analyses were performed to investigate neural mechanisms involved in chronic pain. Pain-related alterations of mediators in the brain may not have been sufficient to produce behavioural deficits in cognition in this experiment but may relate to previous findings. To allow for use of multiple assay techniques (western immunoblotting and HPLC), animals were sacrificed by decapitation rather than by transcardial perfusion.
6.3.4.1 Synaptophysin western immunoblotting

Relative expression of synaptophysin was measured by western immunoblotting in regions considered to be important in pain, cognition and their interaction. The regions measured were: the hippocampus, the frontal cortex, the cerebral cortex, the amygdaloid cortex, the thalamus and the striatum. A representative immunoblot image from the frontal cortex is shown in Figure 6.19. In the majority of cases, there were no hemispheric differences in protein expression and the results for left and right hemispheres were therefore pooled and depicted graphically (Figure 6.20). Where there were differences within or between hemispheres, data for left and right are also presented in a separate figure (Figure 6.21).

![Image of western immunoblot showing synaptophysin (SYP) and β-actin loading controls](image.png)

Figure 6.19: Representative western immunoblot image, showing the bands for synaptophysin (SYP) and β-actin loading controls, for samples from the right (R) and left (L) frontal cortex, for each of the four treatment groups.

Pooled data were analysed by two-way ANOVAs, with surgery and drug as factors. There were no significant main effects of either factor in the hippocampus, cerebral cortex or thalamus. In the PFC, there was a significant main effect of drug ($F_{(1,30)} = 6.51, \ p = 0.016$), and Fisher’s LSD post hoc tests showed that the relative expression of synaptophysin was decreased in both amitriptyline-treated groups (sham and SNL), compared with their respective controls that had received water ($^* p < 0.05$, Figure 6.20(c)). For the pooled data in the amygdaloid cortex, there was a significant surgery × drug interaction ($F_{(1,30)} = 5.38, \ p = 0.027$), and post hoc tests showed a strong trend ($p = 0.055$) for an increase in expression in
Figure 6.20: Relative expression of synaptophysin in discrete brain regions: (a) hippocampus (b) cerebral cortex (c) frontal cortex (d) thalamus (e) striatum (f) amygdaloid cortex. There were no effects of surgery alone on synaptophysin expression in any of the brain regions examined. In the prefrontal cortex (c), amitriptyline was associated with a decrease in the relative expression in both the sham-ami group compared with the sham-water group and in the SNL-ami compared with the SNL-water group (*$p < 0.05$, Fisher’s LSD post hoc tests).

In the amygdaloid cortex (e), there was a trend for an increase in expression in the sham-ami group compared with the sham-water group ($p = 0.055$). Data are expressed as means ± SEM. $n = 8 – 9$. 

233
the sham-ami group compared with the sham-water group (Figure 6.20(e)). There also appeared to be a similar increase in the SNL-water group compared with the sham-water group, but the effect was not statistically significant.

Lateralised data did not display homogeneity of variance when they were normalised to their hemispheric control, and so these data were analysed by Friedman’s ANOVA by ranks and within-hemisphere Kruskal-Wallis tests followed by Mann-Whitney U comparisons. In the amygdaloid cortex, the overall Friedman’s ANOVA was significant ($\chi^2_{(7)} = 16.11, \ p = 0.024$), and Kruskal-Wallis tests revealed a significant effect of group in the right hemisphere ($\chi^2_{(7)} = 9.10, \ p = 0.028$). There was a trend for increased expression of synaptophysin in the right hemisphere compared with the left hemisphere in the SNL-water and the sham-ami groups. Within the right hemisphere, the expression in these groups, and in the SNL-ami group, was significantly higher (*$p < 0.05$) than in the sham-water group (Figure 6.21). These results suggest lateralised effects of both SNL surgery and drug treatment on expression of synaptophysin in the amygdaloid cortex.

Figure 6.21: Lateralised relative expression of synaptophysin in the amygdaloid cortex. There was a trend for expression of synaptophysin to be increased in the right hemisphere compared with the left in SNL-water and sham-ami groups. In the right hemisphere, protein expression was increased by both SNL surgery and amitriptyline treatment, and by the two treatments in combination (*$p < 0.05$ vs. sham-water right, Mann-Whitney $U$ tests). Data are expressed as means ± SEM. $n = 4 - 5$. 

234
6.3.4.2 Assessment of brain and spinal cord monoamine levels using HPLC

Levels of the monoamines noradrenaline, serotonin and dopamine, the dopamine metabolites DOPAC and HVA, and the serotonin metabolite 5-HIAA were measured using the HPLC assay described in Chapter 2. The dopamine turnover (calculated as a ratio of DOPAC or HVA to dopamine) and the serotonin turnover (calculated as the ratio of 5-HIAA to serotonin) were also assessed. The brain regions investigated were the same as for synaptophysin immunoblotting above (hippocampus, frontal cortex, cerebral cortex, amygdaloid cortex, thalamus and striatum). In addition, the levels of monoamines in the dorsal part of the spinal cord (at the level of the L4–L6 lumbar vertebrae) were also measured.

Similar to the synaptophysin expression measurements, there were no hemispheric differences in the majority of cases; the results for left and right hemispheres were therefore pooled and the results are presented in Table 6.2. Selected significant results are also presented in Figures 6.22–6.25. There were relatively few significant alterations in monoamine levels associated with either SNL surgery or amitriptyline administration, or with both treatments in combination. However, in the cerebral cortex, SNL surgery was associated with an increase in levels of dopamine compared with sham-water controls (Kruskal-Wallis test: $\chi^2_{(3)} = 10.22$, $p = 0.017$, Mann-Whitney U test: $*p < 0.05$, sham-water vs. SNL water; see Table 6.2 and Figure 6.22). There was a similar trend in the amitriptyline-treated rats but this did not reach the level of statistical significance ($p = 0.086$). Similarly, DOPAC was found to be increased in the SNL-water group compared with the sham-water group following a two-way ANOVA (significant main effect of surgery, $F_{(1,26)} = 5.15$, $p = 0.032$) and Fisher’s LSD post hoc test (***$p < 0.01$, sham-water vs. SNL-water). This SNL-associated increase appeared to be attenuated by administration of amitriptyline as the level of DOPAC was decreased in the SNL-ami group compared with the SNL-water group ($^+p < 0.05$) and the SNL-ami group did not differ from the sham-water
control group. Furthermore, surgery was associated with a trend for a decrease in dopamine turnover both as a ratio of DOPAC to dopamine and as a ratio of HVA to dopamine. In both cases, data were analysed by two-way ANOVA with surgery and drug as factors. The main effect of surgery was approaching the level of significance for both the DOPAC/dopamine ratio ($F_{(1,25)} = 3.53, p = 0.072$) and the HVA/dopamine ratio ($F_{(1,17)} = 3.65, p = 0.073$).

Figure 6.22: Levels of monoamine transmitters, metabolites and turnovers in the cerebral cortex: (a) Dopamine (b) DOPAC (c) Dopamine turnover. Levels of dopamine and DOPAC were significantly increased in the SNL-water group compared with the sham-water group ($^{*}p < 0.05$, $**p < 0.01$). The increase in DOPAC was attenuated by amitriptyline treatment ($^{+}p < 0.05$, SNL-ami vs. SNL-water), and there was a trend for similar reversal in the level of dopamine following amitriptyline treatment. SNL was also associated with a trend for decrease in dopamine turnover (DOPAC/dopamine and HVA/dopamine ratios). Data are expressed as means ± SEM. $n = 6 - 9$. 

236
### Table 6.2: HPLC Results

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<th>Treatment Group</th>
<th>Sham-water</th>
<th>SNL-water</th>
<th>Sham-ami</th>
<th>SNL-ami</th>
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<td>Noradrenaline</td>
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<td>510.9 (36.2)</td>
<td>477.8 (32.2)</td>
<td>494.6 (33.8)</td>
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<tr>
<td>Dopamine</td>
<td>47.3 (7.4)</td>
<td><strong>249.0 (77.5)</strong> *</td>
<td>52.9 (9.7)</td>
<td>103.2 (27.2)</td>
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<td>750.1 (39.5)</td>
<td>698.5 (16.1)</td>
<td>777.2 (71.2)</td>
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<tr>
<td>DOPAC</td>
<td>58.1 (7.5)</td>
<td><strong>117.4 (19.6)</strong> **</td>
<td>64.3 (8.9)</td>
<td><strong>70.3 (12.6)</strong> *</td>
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<tr>
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<td>74.8 (5.9)</td>
<td>73.6 (3.5)</td>
<td>76.5 (6.0)</td>
<td>74.2 (6.6)</td>
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<tr>
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Table 6.2: continued from previous page.

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<th>Sham-ami</th>
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<td>1000.9 (96.4)</td>
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<td>ND</td>
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<td><strong>0.68 (0.07)</strong> **</td>
<td>0.73 (0.08)</td>
<td><strong>0.54 (0.04)</strong> ***+</td>
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Table 6.2: Pooled levels of monoamines, their metabolites and turnovers in discrete brain regions of sham or SNL rats that received chronic treatment with amitriptyline or water. Levels of noradrenaline, dopamine, serotonin, DOPAC, 5-HIAA and HVA are given in ng/g tissue, and are presented as mean values with the SEM in parenthesis. Turnovers are presented as the ratio of monoamine to its metabolite. ND: not detected in sample; N/A: not applicable. ***p < 0.001, **p < 0.01, *p < 0.05 vs. sham-water; +p < 0.05 vs. SNL-water; #p < 0.05 vs. sham-ami.
Figure 6.23: Levels of monoamine transmitters and metabolites in the thalamus: (a) Dopamine (b) DOPAC. The level of dopamine was significantly increased in the SNL-water group and there was a trend for a similar increase in the level of DOPAC. There was no effect of amitriptyline on the surgery-related increase. Data are expressed as means ± SEM. \( n = 7 − 8 \). (*\( p < 0.05 \) vs. sham-water, #\( p < 0.05 \) vs. sham-ami).

In the thalamus, the concentration of dopamine was also increased in the SNL surgery condition, regardless of amitriptyline treatment (Figure 6.23). A two-way ANOVA of square-root-transformed data revealed a significant main effect of surgery \( (F_{(1,31)} = 11.10, \ p = 0.003) \) and Fisher’s LSD post hoc tests, indicating differences between the sham-water and SNL-water group (*\( p \leq 0.05 \)) and between the sham-ami group and the SNL-ami group (#\( p < 0.05 \)). Again, similar results were observed for the levels of DOPAC in the thalamus. A Kruskal-Wallis test suggested an effect of group \( (\chi^2_{(3)} = 8.79, \ p = 0.032) \) and Mann-Whitney \( U \) test showed that DOPAC was increased in the SNL-ami group compared with the sham-ami group (#\( p < 0.05 \)) and there was a near-significant trend for an increase in the SNL-water group compared with the sham-water group \( (p = 0.064) \).

In the dorsal spinal cord, there was a significant effect of amitriptyline treatment on the on the level of the dopaminergic metabolite DOPAC (two-way ANOVA, main effect of drug: \( F_{(1,61)} = 5.06, \ p = 0.028 \)) (Figure 6.24). The levels of DOPAC were significantly higher in sham animals that had received amitriptyline compared with sham-water controls. There were also surgery-associated alterations in monoaminergic metabolite levels in the dorsal spinal cord. The level of 5-HIAA,
a metabolite of serotonin, was significantly decreased in the SNL-water group compared with the sham-water group (two-way ANOVA, main effect of surgery: $F_{(1,63)} = 5.15, \ p = 0.027, *p < 0.05$). There was also a trend for amitriptyline treatment to reduce the level of 5-HIAA ($p = 0.082$), and amitriptyline administration in SNL rats did not have any modulating effects ($*p < 0.05$, SNL-ami vs sham-water). The turnover of serotonin, assessed as the ratio of 5-HIAA to serotonin, was also decreased following SNL-surgery. A two-way ANOVA revealed a significant main effect of surgery ($F_{(1,57)} = 10.73, \ p = 0.002$) and Fisher’s LSD post hoc tests showed that turnover was lower in surgery conditions regardless of amitriptyline treatment ($***p < 0.001, **p < 0.01$ vs. sham-water, $+p < 0.05$ vs. SNL-water).

In the hippocampus, there was a significant main surgery-drug interaction effect ($F_{(1,28)} = 4.54, \ p = 0.042$) and Fisher’s LSD post hoc tests showed that the level of serotonin was significantly lower in the sham-ami group than in the sham-water group ($*p < 0.05$; Figure 6.25).

Further minor alterations in monoamine levels were observed in the combined SNL-ami group compared with the sham-water group or with the sham-ami group (for example, HVA/dopamine turnover in the frontal cortex, 5-HIAA-serotonin turnover in cerebral cortex, DOPAC in the thalamus and both noradrenaline and serotonin in the spinal cord (see Table 6.1), which may suggest that the effects of amitriptyline differ in the presence or absence of a neuropathic pain model; however, this hypothesis warrants further investigation.

Lateralisated data were also analysed similar to the synaptophysin western blot data. However, these analyses were limited in their power to detect significant differences due to the associated reduction in the sample size ($n = 4 – 5$). There was only one example of significant lateralisation (presented in Figure 6.26 below). In the amygdaloid cortex, the level of HVA was analysed by three-way ANOVA with surgery, drug and hemisphere as factors. There was a significant main effect of hemisphere ($F_{(1,24)} = 4.46, \ p = 0.045$), and Fisher’s LSD post hoc tests showed
that the level of HVA was significantly higher in the contralateral right hemisphere of the SNL-water group compared with the left hemisphere ($p < 0.05$). Within the right hemisphere, SNL-surgery was associated with an increase in the level of HVA ($p < 0.05$, SNL-water vs. sham-water) and there was a trend for this effect to be reversed by amitriptyline ($p = 0.062$, SNL-ami vs. SNL-water).

Figure 6.24: Levels of monoamine transmitters, metabolites and turnovers in the dorsal spinal cord: (a) DOPAC (b) 5-HIAA (c) serotonin turnover. Amitriptyline treatment in the sham group was associated with an increase in DOPAC. SNL surgery, irrespective of drug treatment, was associated with a decrease in 5-HIAA. SNL was also associated with a decrease in serotonin turnover (5-HIAA/serotonin), an effect enhanced by amitriptyline. Data are expressed as means ± SEM. $n = 14−18$. (**$p < 0.001$, *$p < 0.01$, *$p < 0.05$ vs. sham-water, +$p < 0.05$ vs. SNL-water).
Figure 6.25: Levels of serotonin (5-HT) in the hippocampus. The level of serotonin was decreased in the sham-ami group compared with the sham-water group (*p < 0.05, Fisher’s LSD post hoc test), an effect not present in the SNL-ami group. Data are expressed as means ± SEM. n = 8.

Figure 6.26: Lateralised levels of HVA in the amygdaloid cortex. The levels of HVA was significantly higher in the right hemisphere than in the left in the SNL-water group (Fisher’s LSD post hoc test: $$p < 0.05$$, right vs. left). Within the right hemisphere, protein expression was increased by SNL surgery compared with the sham-water group (Fisher’s LSD post hoc test: *p < 0.05, SNL-water vs. sham-water). Data are expressed as means ± SEM. n = 4 – 5.
6.4 Discussion

In the present study, SNL surgery was associated with characteristic neuropathic pain-like behaviours of mechanical allodynia, thermal hyperalgesia and cold allodynia. The expression of thermal hyperalgesia (but not cold or mechanical allodynia) was partially attenuated by amitriptyline chronically administered in drinking water at a dose of $14.5 \pm 0.2 \text{ mg/kg per day}$. There was no effect of SNL surgery on performance in the novel object recognition task or in the Morris water maze, including the reversal task. Thus, the behavioural results did not replicate those of the previous experiment (Chapter 5), where SNL-associated deficits in novel object recognition and water maze reversal learning were observed. Post-mortem analyses of monoamine levels and synaptophysin protein expression were carried out to address the secondary aims referred to in Section 6.1 above. Amitriptyline treatment was associated with effects in expression of synaptophysin in some discrete brain regions, but SNL surgery per se was not associated with any effects on synaptophysin expression in the hippocampus or frontal cortex, contrary to the immunohistochemistry results presented in Chapter 5. There was, however, a lateralised increase in synaptophysin in the contralateral amygdaloid cortex in SNL rats compared with the ipsilateral region and compared with sham-water controls. A similar increase was observed with amitriptyline treatment in sham rats but not in SNL rats that received amitriptyline. Amitriptyline did not dramatically affect the levels of monoamines in the brain or spinal cord tissue measured 70 days after commencement of the chronic dosing regime. SNL surgery was found to alter mainly dopaminergic transmitter and metabolite levels, and was associated with increases in the levels of dopamine and DOPAC in both the cerebral cortex and the thalamus. In some cases this increase was attenuated by the administration of amitriptyline in SNL rats. SNL surgery also decreased levels of the serotonin metabolite 5-HIAA, and serotonin turnover in the spinal cord.

The average fluid intake and dose of amitriptyline received were similar to previous studies in which amitriptyline was administered chronically in the drinking
water (Esser et al., 2001; Yau et al., 2002). The fact that there was a difference between sham and SNL rats in the dose of amitriptyline consumed, but not in the amount of fluid consumed, relates to the difference in body weight between the SNL-ami and SNL-water groups shown in Figure 6.4. Both SNL and amitriptyline had the effect of decreasing body weight in their own right, and body weight was further decreased when the two treatments were combined. Due to the large number of time points involved, the error in the dose calculation was decreased, thereby increasing the chances of registering a statistically significant difference between groups. However, the range of doses over the entire study in both the sham-ami group and the SNL-ami group was 8–17 mg/kg per day.

The attenuation of thermal hyperalgesia following administration of amitriptyline in SNL rats suggests that amitriptyline had an antihyperalgesic effect in the SNL model of neuropathic pain, a result which is in agreement with previous studies in this and other models (Esser et al., 2001; Esser and Sawynok, 2000; Pradhan et al., 2010). The modality-specific nature of the effect is also similar to findings reported in the literature. Amitriptyline was consistently found to reverse thermal hyperalgesia but not mechanical allodynia in rodent models of neuropathic pain (Arsenault and Sawynok, 2009; Esser et al., 2001; Pradhan et al., 2010). In the case of cold allodynia, Pradhan et al. (2010) did show that cold allodynia associated with the CCI model was sensitive to treatment with amitriptyline but the treatment did not significantly reverse the allodynia. In addition to reversal of thermal hyperalgesia in the hindpaw ipsilateral to nerve-injury, Esser et al. (2001) also found that amitriptyline was associated with hyperaesthetic responses to mechanical and thermal stimuli in the contralateral paw. In the present experiment, similar trends were observed only at discrete time points post surgery (Hargreaves test: $p = 0.068$, day 1; von Frey test: $p = 0.083$, week 1; acetone-drop test: $p = 0.017$, week 2. Significance values quoted for SNL-ami vs. SNL-water, Mann-Whitney $U$ tests; see Appendix B, Section B.3) and not at later time points, as reported previously (Esser et al., 2001).
In the novel object recognition task, all rats exhibited a preference for the novel object compared with the familiar object in Exposure 2, indicating that recognition memory was intact and that it was not sensitive to the effects of either amitriptyline or SNL surgery. This finding is surprising given the results presented in Chapter 5, which demonstrated a clear lack of preference for the novel object in the SNL group compared with the sham controls. Sham and SNL groups (Chapter 5 experiment) were treated in an identical manner to the SNL-water and sham-water groups in the present experiment. It is also perhaps surprising that there was no effect of amitriptyline per se on the novel object recognition task. Amitriptyline has been shown to be associated with impaired cognitive function in both clinical (Amado-Boccara et al., 1995; Spring et al., 1992) and preclinical (Naudon et al., 2007; Pavone et al., 1997) settings. However, other studies have shown that amitriptyline had no effect on object discrimination at low doses (2 mg/kg) (Orsetti et al., 2007). An apparent side-bias was again evident in the SNL-water group; however, it was in the opposite direction to the previous study, with animals in this experiment tending to preferentially explore the object on the right regardless of its familiarity.

In the Morris water maze, although the path lengths recorded during acquisition training were broadly similar, the learning curves were less steep than in the previous experiment (Chapter 5), suggesting that the task was not learned as well in the present cohort of rats. A main effect of time, normally indicative of a reduction in path length over the days of acquisition, was not observed in either of the surgery group, which may suggest a surgery-related impairment of acquisition. However, there were no significant differences between the groups at any of the time points. Peripheral nerve injury (transection if the L5 spinal nerve) has been shown previously to impair spatial acquisition in the traditional water maze task (Hu et al., 2010), and this impairment was found to be reversed by amitriptyline. In the present study however, there was no modulatory effect of amitriptyline in this task. For both SNL groups, the percentage distance moved was above the
level of chance in the platform quadrant in the forward probe trial, indicating intact memory of the spatial location of the platform. On the other hand, the percentage distance moved in the platform quadrant was significantly lower in the sham-ami group in the forward probe trial compared with the sham-water group, suggesting a drug-related impairment of spatial memory in this group.

There was no evidence of acquisition of the reversal task as the percentage distance moved in the old platform location did not decrease over time. The percentage distance moved in the new location did increase slightly, but in the sham-ami group only. Thus, these results are contradictory to the findings in Chapter 5, and to those of Leite-Almeida et al. (2009). The performance of the sham-water group in reversal training, and also in the reversal probe trial, was particularly poor. This reduction in basal performance makes it extremely difficult to detect surgery- or drug-related effects in the reversal task.

The increase in swim speed associated with amitriptyline treatment (see Appendix B, Section B.3) was unexpected as it occurred in the sham animals and so was not a consequence of the antihyperalgesic effects. Due to its histaminergic actions, amitriptyline would generally be considered to have sedative effects (Amado-Boccara et al., 1995). It is possible that the increased swim speed relates in some way to the anxiolytic effects of amitriptyline, though this would require further investigation. There have been surprisingly few investigations regarding the effects of amitriptyline on cognitive performance in the water maze. However, chronic amitriptyline was found to reverse age-related cognitive decline (Yau et al., 2002).

The discrepancies in the cognitive behavioural data between this study and that in Chapter 5 are concerning and difficult to explain. To investigate the reasons for these discrepancies, comparisons were made between potentially confounding variables in the common groups across the two studies (sham and SNL in Chapter 5, and sham-water and SNL-water in Chapter 6). The relevant values are summarised in Table 6.3. The precise ages of rats were well controlled: the
Chapter 5 cohort was shipped at 41 weeks of age and habituated for 1 week, with surgery carried out at 43 weeks of age; and the Chapter 6 cohort was shipped at 40 weeks of age and habituated for 2 weeks, with surgery carried at 43 weeks of age. It is possible that even this difference of one week in habituation and daily handling regime had an effect on behavioural outcomes, though it seems unlikely that such a behavioural effect would still be evident in water maze testing which was carried out 46–60 days after the initial habituation and handling period. The administration of amitriptyline in drinking water negated the need for drug injection and additional handling which might have been associated with stress. There was a difference in the weight range of the animals, with the rats in the Chapter 6 cohort being heavier on average than those in the Chapter 5 cohort on arrival (Table 6.3).

There were differences in the relative frequency of sensory testing (see the experiment timelines, Figure 5.1 and 6.2). The rationale for this change was to allow better monitoring of altered sensory responses over the entire study (in Chapter 5, sensory testing was carried out in the first 2 weeks post surgery and at a final time point at the end of the cognitive testing phase). More regular assessment of thermal hyperalgesia in the Hargreaves test was also thought important as amitriptyline has been shown to be antihyperalgesic in this test, but not antiallodynic in the von Frey test. The post-surgery von Frey thresholds were similar in the SNL water groups in both studies, though the baseline threshold was slightly lower in the Chapter 6 cohort of rats (Table 6.3). In the Hargreaves test, despite the increase in the frequency of testing, the pre- and post-surgery paw-withdrawal latencies, and the relative change post surgery compared with baseline, were similar across studies in both sham-water and SNL-water groups (Table 6.3). A significant reduction in withdrawal latency associated with SNL was observed on day 8 in Chapter 5 but not on day 7 (week 1) in Chapter 6, where the SNL-induced hyperalgesia was expressed from week 2 onwards. Thus, there may have been subtle differences in temporal expression of neuropathic pain-like
behaviours between the two studies. The number of responses to acetone application across the studies was also broadly similar, with the group in Chapter 6 perhaps a little more responsive to acetone application post SNL (change in the number of responses from baseline was 9 for Chapter 5 vs. 15 for Chapter 6).

There were also some important differences in the cognitive behavioural test regimes. In the Chapter 5 experiment, rats were tested in the passive-avoidance paradigm, but this test was excluded from the present study. The novel object recognition test was carried out at a later time point post surgery in the experiment in Chapter 6 (Table 6.3). During the novel object habituation, the total distance moved by both sham and SNL rats was lower in the Chapter 6 experiment than in the Chapter 5 one (Table 6.3). This may be important in the context of the experiment as it may reflect a general decrease in the motivation to explore. On the other hand, the total exploratory measure (summed duration of sniffing and rearing) was actually higher in Chapter 6 than in Chapter 5 (Table 6.3), which would suggest the opposite. There were no obvious differences in time the rats spent grooming. The discrimination ratios were similar for the two objects in Exposure 1: sham and SNL groups in both studies explored the two objects equally, as expected, yielding discrimination ratios of approximately 0.5. In the water maze cued test, the basal latency to the platform was similar across studies (Table 6.3). However, the rate of decrease in the latency to the platform appeared to be increased in the sham and SNL groups in Chapter 6 compared with those in Chapter 5, and by the end of the four cued test trials the latency was much lower in the Chapter 6 cohort than in Chapter 5 cohort (Table 6.3). It would appear from these data that the rats used in Chapter 6 had more effectively learned the goal of using the platform as an escape route. However, the path length to get onto the platform on day 1 of acquisition was similar in the two studies, with the exception of the SNL group in Chapter 6 whose path length was shorter than the equivalent group in Chapter 5 (Table 6.3). The path length at the end of the acquisition phase was also similar in both studies, indicating comparable ability
to perform the task. In addition, swim speeds were similar for the two studies at the beginning of the acquisition phase (Table 6.3).

Overall, differences in behaviour or other confounding variables across the studies were minimal. However, in aged rats the influence of even very modest changes in elements such as habituation and frequency of sensory testing could possibly impact on cognitive behaviour and may therefore be responsible for the differences observed in novel object recognition and water maze reversal task performance. It is also possible, given the age of the rats, that their baseline cognitive performance is subject to greater inter-individual variability and thus the power to detect significant differences in behaviour was reduced by the slightly smaller sample sizes employed in this experiment. However, there is little indication of such an effect in the studies described herein, given that the standard errors of the mean for behavioural data were broadly similar for young rats (Chapters 3 and 4) and mid-aged rats (Chapters 5 and 6).

There were no significant effects of SNL surgery or amitriptyline administration, alone or in combination, on relative synaptophysin expression in the hippocampus, the thalamus, the cerebral cortex or the amygdaloid cortex. Amitriptyline decreased the expression of synaptophysin in the prefrontal cortex, and SNL surgery and amitriptyline administration had the combined effect of increasing synaptophysin expression in the striatum. In the experiment described in Chapter 5, SNL surgery was associated with an increase in the expression of synaptophysin in the prelimbic region of the prefrontal cortex and in the CA1 region of the hippocampus, while findings by Ren et al. (2011) suggest that SNL surgery is associated with a decrease in synaptophysin-positive terminals in the CA1. The lack of main effects of surgery observed in the present study appears to contradict the findings of both of these previous investigations. However, both in Chapter 5 and in the paper by Ren et al. (2011), expression of synaptophysin was measured by immunohistochemistry, whereas the analytical technique used in the present study was western immunoblot. This method involved homogenising gross-dissected tis-
sue from each of the isolated brain regions, and as such the assessment was associated with a much lower spatial resolution than that for immunohistochemistry. It is possible, given the complexity of the cell types in the brain regions investigated, that while very few net effects were observed in whole tissue, subregion-specific differences may have been present. Few studies have investigated the effect of amitriptyline administration on expression of synaptophysin. However, Drigues et al. (2003) showed that expression of synaptophysin mRNA was upregulated in the hippocampus of rats treated with oral amitriptyline for 10 days. An increase in synaptophysin protein in the hippocampus might also therefore be expected following chronic administration of amitriptyline, though such an increase was not observed in the present study. The amitriptyline-associated decrease observed in the frontal cortex may be specific to that region, or the expression may differ between the levels of mRNA and protein.

The concentrations of monoamines measured in discrete brain regions were consistent with those measured previously in our laboratory (see, for example, Burke et al., 2010; Ford et al., 2008; Simpson et al., 2012). Monoaminergic concentrations in the spinal cord were also similar to those reported previously in the literature (Nakajima et al., 2012; Skup et al., 2007). Pain-related alterations in monoamine levels, particularly increases in dopamine and its metabolite DOPAC, were observed in the cerebral cortex and the thalamus in the present study. There was also a decrease in dopamine turnover in the cerebral cortex associated with SNL surgery. In the case of DOPAC, in the cerebral cortex, this effect was reversed by concurrent amitriptyline administration. This result may suggest that supraspinal alterations in monoamine levels or metabolism maintain peripheral neuropathic pain, with their modulation resulting in an antihyperalgesic effect. SNL surgery was also associated with a decrease in the level of serotonin in the dorsal spinal cord and a decrease in serotonin turnover. The level of HVA expression was increased in the contralateral amygdaloid cortex of SNL rats compared with the ipsilateral side, and also with the contralateral side in the sham-water
control group. Thus, alterations in monoamines associated with SNL surgery may occur in a laterised manner in some brain regions. Amitriptyline treatment tended to have little effect or even to reduce the level of monoamines both at the spinal and supraspinal level (with the exception of DOPAC in the spinal cord, which was increased). These results seem counter-intuitive given that amitriptyline is known to block the reuptake of both serotonin and noradrenaline.

Despite the established role of the monoamine system in pain perception, characterisation of supraspinal monoamine levels in the SNL model has not been carried out previously. The main effects observed herein were in the dopaminergic system. Abnormal dopamine neurotransmission has been demonstrated previously in clinical chronic pain conditions (Wood et al., 2009, 2007b), and there is also evidence for altered dopaminergic activity in the PAG, thalamus, PFC and orbitofrontal cortices in models of pain and pain-related cognitive impairment (Finn et al., 2006; Ford et al., 2008; Pais-Vieira et al., 2009b). Though cognitive deficits were not manifested in the behavioural tests in the present study, the finding of altered levels of dopamine (and the dopamine metabolite DOPAC) in the thalamus and cerebral cortex may be important in the context of pain, cognition and pain-related cognitive impairment. The dopaminergic system is heavily involved in cognitive processes such as learning and extinction, working memory, sustained attention, attentional set-shifting and reversal learning (Robbins and Arnsten, 2009). Normal cognitive function requires the correct balance of dopaminergic signalling, and both increases and decreases in transmitter levels may result in impairments (Robbins and Arnsten, 2009; Lyon et al., 2012). Dopaminergic dysfunction is thought to underlie or exacerbate the cognitive deficits observed in a number of neurological disorders including schizophrenia (Cohen and Servan-Schreiber, 1992; Kapur, 2003), attention-deficit/hyperactivity disorder (ADHD) (Castellanos and Tannock, 2002), Alzheimer’s disease (Kemppainen et al., 2003, 2000; Pizzolato et al., 1996), Parkinson’s disease (Nagano-Saito et al., 2004) and obsessive-compulsive disorder (OCD) (Westenberg et al., 2007). In addition, cog-
nitive impairments are common in affective disorders such as depression and anxiety, the pathophysiology of which also involves dopamine dysfunction (Jarcho et al., 2012). Dopaminergic signalling in the thalamus and cerebral cortex has not been investigated extensively in relation to cognition. However, both regions are connected with important cognitive-associated brain regions such as the hippocampus and prefrontal cortex, as well as other components of the limbic system. It is also noteworthy that the thalamus and cortex are themselves highly interconnected, and dopamine in the thalamocortical circuit is involved in consciousness, a process which would likely have an effect on cognitive functioning. Thus, altered dopamine signalling in the brain can affect the processing systems involved in pain and cognition, and consequently the changes in dopamine levels observed in this experiment may warrant further investigation.

The fact that monoamine levels were unchanged or decreased in the spinal cord in SNL rats is contrary to a previous report which showed that serotonin and noradrenaline were increased in the spinal cord of SNL rats (Nakajima et al., 2012). However, this difference may be explained by differences in the harvesting of spinal cord tissue. In the present study, the lumbar spinal cord in the region of L4–L6 was collected using a similar procedure to that of Nakajima et al. (2012); however, we also medially divided the cord into dorsal and ventral segments and discarded the ventral portion. Thus, the monoamine content of only the dorsal spinal cord was measured in the current experiment. It is also important to note that the assay used in the present study measures the tissue concentration of monoamines, including intracellular and extracellular levels. As amitriptyline blocks the reuptake of monoamines such as noradrenaline and serotonin, it may be more informative to measure the extracellular, synaptic levels of monoamines. Future studies using microdialysis could be used to determine whether extracellular monoamine levels are altered following amitriptyline administration in the SNL model.

The observation that amitriptyline treatment was associated with a significant
attenuation of the SNL-induced thermal hyperalgesia, but did not increase spinal or supraspinal concentrations of monoamines, suggests that the mechanism of action of amitriptyline involves non-monoaminergic targets. Furthermore, oral amitriptyline in the rat is subject to high levels of first-pass metabolism (Bae et al., 2009) and therefore its antihyperalgesic action may be exerted at a lower dose, and at peripheral targets such as sodium channels.

One major limitation of the neurochemical measures in this study (in particular the measurement of monoamines) is that no adjustment was made for the relatively high number of statistical comparisons. While calculations such as the Bonferroni correction may decrease the likelihood of obtaining false positive results, the level of statistical significance required may be too conservative to detect subtle surgery or drug effects.

The results of the present experiment differ from the results presented in Chapter 5, and suggest that the SNL-induced cognitive impairment may be sensitive to subtle alterations in variables such as the sequence of behavioural testing. As such, SNL in mid-aged rats as a model of cognitive impairment in chronic pain may be difficult to reproduce. In spite of the lack of behavioural deficits in cognition, SNL surgery was found to be associated with some regional differences in synaptophysin expression and levels of monoamines in the brain. Further studies are also needed to determine the antihyperalgesic mechanism of amitriptyline.
Chapter 7  Investigation of Cognitive Performance in a Clinical Sample of Chronic Pain Patients

7.1 Introduction

Chapter 1 (Section 1.1.1 and Table 1.1) provides a thorough overview of previous studies that have investigated cognitive functioning in clinical samples of chronic pain patients. The majority of the research reported appears to provide support for an inverse relationship between pain and cognitive functioning. However, a number of important limitations of these studies have been identified, and are discussed in detail in Section 1.1.3. These limitations are mainly due to differences in the pain and cognitive scale materials used, and the heterogeneity of patient participants both in terms of their demographics and pathological conditions.

As illustrated in Table 1.1, there is no obvious pattern of cognitive deficits related to chronic pain in general, with no one cognitive domain impaired more than others, and with the type of impairment varying across disorders. This suggests that the specific type of disorder, or type of pain, may be an important determinant of the cognitive outcome. A number of studies have investigated the relationship between pain and cognitive function in particular disorders (for example, migraine, FM or diabetic neuropathy, see Table 1.1). Less emphasis has been placed on examining specific pain types, irrespective of their aetiology. However, in one study that did compare different pain types, attention was found to be impaired to a similar extent in rheumatoid arthritis, musculoskeletal pain and FM patients compared with healthy controls (Dick et al., 2002). On the other hand, emotional decision making was impaired in lumbar spinal or radicular pain of the lower back, but not in CRPS (Apkarian et al., 2004a), and general
cognitive functioning was poorer in neuropathic pain patients than in patients with a diagnosis of mixed neuropathic and nociceptive pain (Povedano et al., 2007). A full characterisation of the cognitive performance in specific pain types may allow more accurate comparisons to be made between studies (and differing experimental conditions) and may provide a better insight into the cause of pain-related deficits, if present.

The studies presented in this dissertation have focused primarily on neuropathic pain. Although previous investigations of cognition in chronic pain have included neuropathic pain patients within the tested sample, few have examined performance in neuropathic pain per se. Povedano et al. (2007) demonstrated cognitive impairment in a neuropathic pain cohort compared with the reference population on the MMSE. The lack of a matched comparator group is a clear weakness of this study. Furthermore, the MMSE is a very general measure of function, and the cognitive domains measured are poorly defined; it is generally used as a screening tool for the detection of cognitive impairment, and not for measurement of specific cognitive deficits. A back-translational approach involving the preclinical literature, including the studies presented herein, would also suggest deficits in cognitive function related to neuropathic pain. Impairments in spatial learning and memory, recognition memory, and cognitive flexibility have been demonstrated in rodent models of neuropathic pain (see Table 1.3).

On the potential role of age on pain and cognition, a number of studies (Karp et al., 2006; Weiner et al., 2006; Buckalew et al., 2010) have found pain-related deficits specifically in older pain patients, consistent with commonly reported effects in younger patient cohorts, but these studies did not address directly the question of whether age mediates the relationship between pain and cognition. However, a positive relationship or an improvement in executive function associated with increases in reported pain has been observed in older adults (Oosterman et al., 2009) and in older Alzheimer’s disease patients (Scherder et al., 2008), suggesting a more complex effect of age. One recent study examined the interaction
of pain and age on cognitive function (Oosterman et al., 2011) in patients with various types of chronic pain, and found that the interaction made no significant contribution to cognitive task outcomes. Preclinical findings also imply that further investigation of the pain and age interaction in relation to cognition is warranted. Leite-Almeida et al. (2009) demonstrated age-dependent effects of pain on cognition in a rat model of neuropathic pain, while the investigations presented here in Chapters 5-6 provide further evidence for an important role of age.

The goal of the present study was to carry out a clinical investigation of cognitive function in chronic pain, which would address, where possible, limitations associated with previous research, and which would encompass the translational knowledge accumulated throughout the project as a whole. Thus, the chronic pain patient sample was recruited on the basis of the type of chronic pain, with the inclusion criteria requiring a diagnosis of neuropathic pain or radiculopathy associated with radicular pain. The latter type of pain is similar to neuropathic pain but relates specifically to a spinal nerve or its roots. The IASP defines radiculopathy as “objective loss of sensory and/or motor function as a result of conduction block in axons of a spinal nerve or its roots” and radicular pain as being caused by ectopic activation of that nerve or root (IASP Task Force on Taxonomy, 1994). Patients with neuropathic pain in the form of diabetic neuropathy were, however, excluded. As discussed in Chapter 1, cognitive impairments have been demonstrated in diabetes and epidemiological studies have shown an association between diabetes and both vascular dementia and Alzheimer’s disease (Ott et al., 1996). However, the relative contribution of pain to these deficits is unclear, compared with other effects of diabetes in the brain including cerebrovascular changes and toxicity associated with hyperglycaemia (Gispen and Biessels, 2000). An effort was made to recruit patients covering a broad age range in order to effectively investigate the influence of age on pain and cognitive function. For operational reasons, however, patients were recruited from an outpatient pain management clinic and therefore the sample did not include very elderly participants (the oldest
patient participant was aged 64 years). The contribution of age was investigated statistically using regression modelling. Different aspects of pain, including intensity and disabling effect, were quantified in the patient group.

Participants with a clinical diagnosis of depression or anxiety were excluded, as these conditions commonly coexist with pain and may influence cognitive function (Hindmarch, 1998; Austin et al., 2001; Brown et al., 2002; Castaneda et al., 2008). Unfortunately, despite efforts to recruit patients who were medication-free, a limitation of the present study is that the majority of patients (79%) were receiving analgesic treatment for the management of their pain at the time of testing. The possible influence of analgesic medications on cognitive function is discussed in Chapter 1. Attempts were made to control for this variable statistically, and also to compare different classes of analgesics; however, this level of analysis was within the patient group only and the conclusions may be confounded by the small sample size. Patients were compared to a control group, matched by age and gender, and the potentially confounding effects of a number of participant characteristics, such as the number of years of education completed and recent consumption of caffeine, nicotine and alcohol, were also considered.

Participants were exposed to a comprehensive battery of cognitive tests. The individual measures were carefully chosen in consultation with the supervisors and with expert neuropsychologists so as to investigate a variety of cognitive domains. Furthermore, the measures were selected to align broadly the cognitive domains that were assessed in the preclinical models of neuropathic pain in the earlier chapters herein. The spatial span test was used to assess spatial memory, corresponding to the T-maze alternation and Morris water maze tasks of spatial memory in rodents. Attention was assessed here using the CPT; though attention was not assessed directly in the rodent experiment, object exploration in the novel object recognition task may be associated with attentional processes. Cognitive flexibility/executive-type functioning, as measured by the reversal in the Morris water maze, was assessed using the Wisconsin Card Sorting Test (WCST®).
only exceptions were the verbal memory tasks, where no corresponding preclinical
tasks exist. They were included in the clinical study, however, as they provided an
opportunity to assess learning, delayed memory and recognition memory, which
were also assessed visuospatially in preclinical models. The objective of this ap-
proach was to produce a translational model, whereby inferences could be made
across species regarding the effects of pain on cognition and the potential mecha-
nisms by which these effects may occur.

The specific aims of the study were to investigate the effects of pain (by com-
paring patient and control groups) and the interaction of pain and age on function
across four broad cognitive domains (verbal memory, spatial memory, attention
and executive function) and to determine which pain-related variables, if any, were
predictive of changes in cognitive function.

7.2 Materials and methods

7.2.1 Experimental design

Phase I of this study employed a $2 \times 2$ between-samples design. The first independent
variable was the participant group, which had two levels, chronic pain patient
and control. The second independent variable was age, which was arbitrarily split
into two levels, participants aged 45 years or older and participants aged 44 years
or younger for descriptive purposes. Age (mean-centred) was included as a scale
variable for regression analyses in order to overcome the potential loss of power as-
sociated with splitting measures of individual differences. In Phase II, continuous
measures of pain, along with age, were treated as independent variables.

7.2.2 Participants

As described in Chapter 2, Section 2.2.10, chronic pain patient participants were
recruited from the Pain Clinic at University Hospital, Galway and control partic-
ipants were recruited through placement of advertisements in public places and
in the local and national media. Exclusion criteria were: age less than 18 years, pre-existing cognitive impairment, major psychiatric illness, substance abuse, diabetes, history of epilepsy, seizures or traumatic brain injury, or, in the case of control participants, a history of chronic pain. Inclusion criteria consisted of a sufficient English language ability for the completion of the tests, and, in the case of the patients, a diagnosis of chronic (minimum of 3 months) neuropathic pain or radiculopathy as diagnosed by a specialist pain physician. Seventy-six participants were recruited in total, 38 chronic pain patients and 38 healthy controls, matched on the basis of age and gender (see Table 7.1).

7.2.3 Materials

A brief description of the assessment materials used is provided in Chapter 2, Sections 2.2.11–2.2.15, and copies of the response booklets and/or the assessment tools are presented in Appendix E. The outcome measures recorded from each of the assessment subtests are summarised below.

7.2.3.1 Demographic questionnaire outcome measures

Participants’ age, gender and number of years of education were recorded on a standard demographic questionnaire. Participants were also asked whether they currently smoked cigarettes (or were taking nicotine-replacement therapy), and they were asked, where applicable, to estimate the time since they had last consumed nicotine, alcohol and caffeine, as these drugs are known to act centrally and are associated with alterations in cognitive function. The estimated duration since participants had last consumed nicotine and caffeine were recorded in minutes. In the case of alcohol, a large range of times were recorded and presentation in a single unit was impractical. Instead, the durations were categorised as follows: (1) ≤ 12 hrs, (2) 12 – 24 hrs, (3) 24 – 48 hrs, (4) 48 – 72 hrs, (5) 72hrs – 7 days, (6) 7 days – 1 month, (7) 1 – 6 months. Those who had not consumed alcohol for more than 6 months were classified as “non-drinkers”.  

260
7.2.3.2 Pain questionnaire outcome measures

The following information was gathered from patient participants on a standard form: number of months since diagnosis of pain, number of painful areas (as indicated on the manikin diagram, Figure 2.10), list of current medications and percentage pain relief received from medications in the last 24 hours. Medications were classified by type as: opioid, anticonvulsant, antidepressant, NSAID or other. The number of medications in each category, as well as the total number of medications was recorded. Patient participants also completed the Chronic Pain Grade questionnaire (see Appendix E) which provided scores for present pain intensity, average pain intensity, pain-related disability score, and a chronic pain grade or category reflecting pain severity and interference. Pain intensities were rated on a 10-point Likert scale where 0 was “no pain” and 10 was “pain as bad as it could be”. The average pain intensity was a score from 0–100 calculated from items 3–5 of the Chronic Pain Grade questionnaire as mean (Question 3 + Question 4 + Question 5) $\times$ 10. The pain-related disability score was a score from 0–100 calculated similarly from items 7–9. The disability score was also recoded according to the following scheme: 0–29 $\rightarrow$ 0 points, 30–49 $\rightarrow$ 1 point, 50–69 $\rightarrow$ 2 points, 70+ $\rightarrow$ 3 points. The four possible responses to item 6 were coded 0–3 points (lowest to highest) and added to the recoded disability score to obtain a disability points value in the range 0–6. Chronic pain grades were then defined as: grade 0 pain intensity = 0 and disability points = 0; grade I pain intensity $< 50$ and disability points $< 3$; grade II pain intensity $\geq 50$ and disability points $< 3$; grade III disability points = 3 or 4 regardless of pain intensity; grade IV disability points = 5 or 6, regardless of pain intensity. Self-assessed interference of pain with concentration, memory and problem-solving or decision-making ability was rated on a 10-point Likert scale where 0 was “no interference” and 10 was “extreme interference”.

Pain questionnaires were completed only by chronic pain patients. All participants were presented with the following two items which reflect the IASP definition
of chronic pain and which have been used to screen for chronic pain in a previous large-scale study of chronic pain in Ireland (Raftery et al., 2011):

1. Currently (i.e. over the past few months), have you been troubled by pain or discomfort, either all the time, or on and off?

2. Has this pain lasted for more than 3 months?

If the participant answered “No” to either of these items, they were instructed to proceed to the psychological questionnaires section.

### 7.2.3.3 Psychological questionnaire outcome measures

**Depressive symptoms**

The Patient Health Questionnaire (PHQ, see Appendix E) was used to assess depressive symptoms based on the Diagnostic and Statistical Manual (DSM-IV, American Psychiatric Association (2003)) criteria and provided the following outcome measures: depressive symptom severity score, depression severity classification, depression “diagnosis”, depression type, and presence or absence of functional impairment. The depressive symptom severity score was determined by summing the values in all of the checked boxes on the PHQ form. The depression severity classification was as follows: 0–4 none, 5–9 mild, 10–14 moderate, 15–19 moderately severe, 20–27 severe depression. A “diagnosis” of clinically relevant depressive symptoms was considered if a participant’s answers fell in the highlighted section of the form (see Appendix E) on four or more items (one of which corresponded to Question 1 or Question 2; these questions related to “having little interest or pleasure in doing things” or “feeling down, depressed or hopeless” in the last two weeks – see Appendix E, page 464). The type of depressive disorder present was estimated as a major depressive disorder or other depressive disorder depending on the number of items answered in the highlighted section. Functional

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1While a questionnaire should not be used to diagnose a clinical disorder, the PHQ has a high correlation with diagnosis of likely depression based on gold-standard clinical interview.
impairment was assessed by item 10 of the PHQ and deemed present if the item was endorsed as “somewhat difficult” or greater.

State anxiety
The “state” or current anxiety level questions from the Spielberger State-Trait Anxiety Inventory (STAI-S Spielberger, 1983) were used to calculate a total score for “state” anxiety at the time of testing. Each STAI-S item is classified as anxiety-present (for example: “I feel upset”, or “I feel frightened”) or anxiety-absent (for example: “I feel calm”, or “I feel relaxed”) and is given a score of 1 to 4 (see Appendix E, page 465). The raw score for each anxiety-absent item was reversed to obtain a weighted score = 5 – raw score; for anxiety-present items, weighted scores was taken to be the same as raw scores. The STAI-S total score was determined by adding the weighted scores of all 20 items.

Both the PHQ and the SAI showed good internal consistencies: Chronbach’s $\alpha$ was 0.93 in both cases, well above the recommended threshold of 0.7 (Pallant, 2011).

7.2.3.4 Cognitive function outcome measures
Full-scale IQ was estimated using a dyadic short form of the WAIS®-III, as described in Chapter 2.

The immediate verbal memory was measured using the Logical Memory I subtest from the WMS®-III (Wechsler, 1997c). The test administration is described in detail in Chapter 2, Section 2.2.15. Three outcome measures were obtained using this test: story unit recall, theme unit recall and learning slope. Raw scores for story units and theme units recalled correctly were converted to scaled scores between 1 and 19 using the WMS-III Administration and Scoring manual (Wechsler, 1997d). The learning slope was calculated as the difference in the number of story units recalled between the first and second presentation and recall of story B, and was in the range $-25$ to $+25$. Scaled scores between 1 and 19 were obtained similarly for story and theme unit recall in the delayed verbal memory task (Log-
ical Memory II, WMS-III). Recognition was measured on a scale of 0–30 based on participants’ answers to 30 questions relating to stories A and B. Percentage retention was calculated by comparing recall in the delayed memory task to recall the immediate memory task.

Spatial memory was measured using the Spatial Span subtest of the WMS-III), scaled scores (0–19) were obtained from raw scores for both forward and reverse tasks, and a composite scaled score was obtained for the task overall. The test administration is described in detail in Chapter 2, Section 2.2.15.

The CPT-IP task used to measure attention and vigilance, and procedures for its administration are outlined in Chapter 2, Section 2.2.15. The CPT-IP outcome measures were the number of hits (the number of correct responses to identical pairs) the number of false alarms (the number of incorrect responses to similar, but not identical pairs) and the number of random incorrect responses. The D-prime (range 0–4.2), an indication of the rate of hits to false alarms, was also calculated as an index of attention. The D-prime values were converted to both uncorrected and demographically (age and gender) corrected T-scores with the aid of the MATRICS Consensus Cognitive Battery (MCCB) manual (Nuechterlein and Green, 2004). It is important to note that the MCCB normative data are based on an average D-prime value following the administration of the full CPT-IP task which includes blocks of 2-digit, 3-digit and 4-digit stimuli. Due to time constraints, only the 4-digit block of stimuli was presented in this study. As this would be considered the most challenging of the three blocks, the T-scores may underestimate participants’ performance. Furthermore, the demographically corrected normative data included in the MCCB manual are for participants aged 20–59. A total of 7 participants (4 patients and 3 controls) were outside this age range, and thus the sample size is reduced for this outcome measure. As such, analyses were performed for raw D-prime scores and both the uncorrected and corrected T-scores, and results are presented in Table 7.3. The average reaction times to both hits and false alarms during the CPT-IP trial were used as crude
measures of psychomotor speed.

Executive functioning was assessed using the WCST; the test administration method is described in Chapter 2, Section 2.2.15. The computerised version of the WCST (WCST-CV4) used in this study generated a report providing both raw scores and demographically (age and education) corrected standard scores for five outcome measures: percentage errors, percentage perseverative responses, percentage perseverative errors, percentage non-perseverative errors and percentage conceptual level responses. Detailed methods for the calculation of each of these measures are outlined in the Wisconsin Card Sorting Test manual (Heaton et al., 1993). Percentage outcome measures were used to control for differences in the number of trials administered. Raw scores were also computed for the number of categories completed, the number of trials to complete the first category, number of failures to maintain set and a learning-to-learn score (indicative of conceptual efficiency across consecutive categories). The learning-to-learn score can only be computed if three or more categories have been completed; thus, the \( n \) numbers for this measure were \( n = 24 \) in the patient group and \( n = 31 \) in the control group.

### 7.2.4 Procedure

Patient participants were sent an information sheet (Appendix E) and an invitation to participate in the research study at least one week in advance of the assessment. To reduce patient burden, chronic pain patients were invited to complete the assessment on the day of a routinely scheduled appointment at the pain clinic. The testing was done in advance of the clinic appointment to minimise the confounding effects of interventional analgesic treatments. Where patients could not attend on the day of their appointment, but consented to participate, the assessment was scheduled for an alternative date. For control participants, the study was outlined in the public advertisements, and an information sheet was also provided prior to the assessment. All participants signed the informed consent document (see Appendix E), and the test materials were then adminis-
tered in the following order: demographic questionnaire, pain questionnaire, PHQ, SAI, WAIS dyad (digit-symbol coding followed by information), Logical Memory I (Story A, Story B, Story B recall 2), spatial span – forward, spatial span – reverse, CPT-IP (4-digit block), WCST–CV4 and Logical Memory II (Story A, Story B, recognition). Logical Memory II was administered approximately 25–35 minutes after administration of Logical Memory I, and the entire assessment took about an hour and a half to complete on average. At the end of the assessment, participants received an ex gratia payment of €30, in cash, for their participation.

7.2.5 Statistical design and analysis

*Analysis by group (Phase I)*

This first phase of the study tested two hypotheses. The first hypothesis is that chronic pain is associated with impaired cognitive performance on a range of outcome variables; this hypothesis predicted that participants in the patient group would perform poorly compared with the control group on all of the outcome measures. The second hypothesis is that there is an interaction effect between age and chronic pain condition, based on previous literature and our own preclinical findings presented in earlier chapters, and that this would also affect cognitive performance. The precise nature of the interaction effect was, however, difficult to predict. The literature suggests a complex relationship between pain and ageing (for detailed discussion see Chapter 1), which may not follow a linear pattern. The clinical literature indicates lower pain ratings in older chronic pain patients than in younger patients, while preclinical studies appear to demonstrate a peak in pain sensitivity at mid-age compared with both younger and older groups. The sample investigated here consisted of a limited age range, predominantly concentrated around “mid-age”, and it was therefore hypothesised that within this limited age range the effects of pain on the cognitive outcome variables would be greater with increasing age. That is, the performance would be poorer in the patients than in the controls, and this effect would be greater in the group aged 45 years and over.
The first level of analysis was the computation of descriptive and inferential statistics. This was followed by correlation analyses of cognitive outcome measures and participant characteristics aimed at identifying potential covariates (i.e., variables that correlated significantly with the dependent variables). Parametric correlations (Pearson), parametric correlations with one bivariate variable (point-biserial) or non-parametric correlations (Spearman Rho) were performed as appropriate. As described above, the study employed a two-by-two between-subjects design, but factorial analysis of covariance (ANCOVA) was not suitable for testing the hypotheses as covariates were not compatible with the assumptions of ANCOVA as detailed by Tabachnick and Fidell (2007). Furthermore, the splitting of the continuous age variable into two groups may have been associated with a reduction in power and in effect size (MacCallum et al., 2002). Thus, hierarchical multiple regression was used to test the hypotheses. Multiple regression is used to explore the relationship between a continuous dependent variable and various independent, or predictor, variables. Hierarchical multiple regression involves entering these variables in a predetermined sequence of steps or blocks, which allows investigation of the effects of specific independent variables of interest, while statistically controlling for others. For these analyses, the groups were coded as $-1$ (patient group) and $+1$ (control group), age was mean-centred, and an interaction term ($\text{group} \times \text{age}$) was calculated. The purpose of centring was to minimise multicollinearity, which occurs when independent variables are highly correlated (arising, in this case, from entering both the variables and their interaction into the regression equation). Participant-characteristic variables that were correlated with the cognitive outcomes were entered into the first block of the regression model, thereby reducing their potential to mask the effects of the independent variables. Values for significance levels and $\beta$ coefficients quoted in the text are for the overall model.
Analysis by pain variable (Phase II)

The second phase of the study tested the hypothesis that specific pain variables (i.e., pain intensity, average pain intensity, pain-related disability score, chronic pain grade, number of painful areas and pain chronicity) are good predictors of cognitive outcomes in the pain patient group. As in Phase I, an interaction effect between pain variables and age was also hypothesised, and this was tested in the analyses. The pain variables investigated were present pain intensity, average pain intensity, pain-related disability score, chronic pain grade, number of painful areas and pain chronicity (number of months since diagnosis of pain). Additional patient characteristics investigated as potential covariates were the presence or absence of medication (and different medication sub-groups), total number of medications, percentage pain relief from medications, and self-assessed cognitive function. Descriptive and inferential statistics relating to pain information (for patient participants only, $n = 38$) were calculated. Correlation matrices, using the patient group data only, were constructed to identify potential covariates. Each of the pain variables was centred, and interaction terms with mean-centred age were calculated. Hierarchical regressions were again used to test the hypotheses.

7.3 Results

7.3.1 Phase I: Analysis by group

7.3.1.1 Descriptive and inferential statistics

Demographic and psychological descriptors of pain patient and control groups are presented in Tables 7.1 and 7.2. Box plots, normality plots and inferential tests of normality (Shapiro-Wilk tests) were used to examine the distribution of the potential covariate measures (not shown), and $t$-tests, Mann-Whitney $U$ tests or $\chi^2$ tests were used to compare these measures across groups as appropriate to the assumptions of these tests.
CHAPTER 7. COGNITIVE PERFORMANCE IN CHRONIC PAIN PATIENTS

<table>
<thead>
<tr>
<th></th>
<th>Control Total (Male, Female)</th>
<th>Chronic pain Total (Male, Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of participants</td>
<td>38 (16, 22)</td>
<td>38 (16, 22)</td>
</tr>
<tr>
<td>Mean (standard deviation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>44.2 (10.4)</td>
<td>45.6 (9.9)</td>
</tr>
<tr>
<td>Duration since last consumption of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine (hrs)</td>
<td>3.1 (6.6)</td>
<td>1.6 (3.5)</td>
</tr>
<tr>
<td>Caffeine (hrs)</td>
<td>5.8 (8.3)</td>
<td>5.3 (10.7)</td>
</tr>
<tr>
<td>Alcohol (category value)†</td>
<td>4.8 (1.5)</td>
<td>4.0 (1.5)</td>
</tr>
<tr>
<td>Years of education</td>
<td>15.2 (3.0)</td>
<td>13.8 (3.8)</td>
</tr>
<tr>
<td>Number of participants in each age classification</td>
<td>17 / 21</td>
<td>22 / 16</td>
</tr>
<tr>
<td>Percentage of participants in group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of participants who smoke</td>
<td>18.4</td>
<td>50.0</td>
</tr>
</tbody>
</table>

† See discussion in Section 7.2.3.1.

Table 7.1: Demographic information.

Basic between-group comparisons were made in order to identify any differences between the patient and control samples on variables of interest. There were no significant differences between patient and control groups in age or age group (defined as 45+ years and < 45 years), gender, years of education and duration since last consumption of caffeine or nicotine. There were, however, significantly more smokers in the patient group than in the control group, and patients exhibited higher depressive symptom scores, increased depression-related functional impairment and greater levels of state anxiety than controls. The duration since last consumption of alcohol was slightly, but significantly, longer in the patient group than in the control group when the averages of the categories\(^2\), defined previously in Section 7.2.3.1, were compared (controls: 4.0 ± 1.5 days, patients: 4.8 ± 1.5 days).

\(^2\) (1) ≤ 12 hrs, (2) 12 – 24 hrs, (3) 24 – 48 hrs, (4) 48 – 72 hrs, (5) 72hrs – 7 days, (6) 7 days – 1 month, (7) 1 – 6 months.
A preliminary comparison of the dependent variables across groups (patient and control) indicated consistently lower outcome scores in the patient group, or higher scores on reverse scales (number of false alarms and number of random responses in the CPT-IP, and raw scores for % errors, % perseverative responses, % perseverative errors, % non-perseverative errors, number of trials to complete the first category and failure to maintain set in the WCST); the only exception was % retention in the delayed verbal memory task which was marginally higher in the patient group compared with the control group (see Table 7.3). Although some of these differences failed to reach statistical significance, the trends in the data appear to be in line with the first hypothesis that patients would perform poorly compared with controls.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Chronic Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated full scale IQ</td>
<td>103.16 (13.10)</td>
<td>87.61 (14.83)</td>
</tr>
<tr>
<td><strong>Verbal Memory:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Immediate:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unit Recall</td>
<td>11.58 (2.77)</td>
<td>8.61 (3.10)</td>
</tr>
<tr>
<td>Theme Recall</td>
<td>10.76 (2.42)</td>
<td>8.45 (3.11)</td>
</tr>
<tr>
<td>Learning Slope</td>
<td>4.89 (2.70)</td>
<td>3.97 (2.73)</td>
</tr>
<tr>
<td><strong>Delayed:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unit Recall</td>
<td>11.24 (2.66)</td>
<td>9.24 (2.60)</td>
</tr>
<tr>
<td>Theme Recall</td>
<td>10.58 (2.89)</td>
<td>9.24 (2.74)</td>
</tr>
<tr>
<td>Recognition</td>
<td>26.37 (2.30)</td>
<td>25.11 (3.14)</td>
</tr>
<tr>
<td>% Retention</td>
<td>77.34 (15.10)</td>
<td>77.78 (13.31)</td>
</tr>
<tr>
<td><strong>Spatial Memory:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>9.87 (3.35)</td>
<td>8.42 (2.94)</td>
</tr>
<tr>
<td>Reverse</td>
<td>11.03 (2.81)</td>
<td>9.42 (3.59)</td>
</tr>
<tr>
<td>Total</td>
<td>10.45 (3.06)</td>
<td>8.66 (3.40)</td>
</tr>
<tr>
<td><strong>Attention:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hits</td>
<td>23.50 (3.90)</td>
<td>22.16 (3.99)</td>
</tr>
<tr>
<td>False Alarms</td>
<td>6.47 (3.70)</td>
<td>8.42 (5.20)</td>
</tr>
<tr>
<td>Randoms</td>
<td>2.11 (2.89)</td>
<td>3.34 (4.10)</td>
</tr>
<tr>
<td>CPT D Prime</td>
<td>1.29 (0.30)</td>
<td>1.11 (0.38)</td>
</tr>
<tr>
<td>CPT T-Score</td>
<td>32.03 (11.27)</td>
<td>27.79 (10.49)</td>
</tr>
<tr>
<td>CPT T-Score Corrected</td>
<td>32.37 (11.06)</td>
<td>28.97 (10.76)</td>
</tr>
<tr>
<td><strong>Psychomotor speed:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hits reaction time (s)</td>
<td>559.59 (665.40)</td>
<td>544.38 (75.34)</td>
</tr>
<tr>
<td>False alarm reaction time (s)</td>
<td>566.51 (126.00)</td>
<td>562.72 (112.69)</td>
</tr>
<tr>
<td><strong>Executive Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Raw scores):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Errors</td>
<td>28.95 (18.42)</td>
<td>35.42 (19.01)</td>
</tr>
<tr>
<td>% Perseverative Responses</td>
<td>16.03 (14.31)</td>
<td>21.50 (18.25)</td>
</tr>
<tr>
<td>% Perseverative Errors</td>
<td>14.11 (11.07)</td>
<td>18.87 (13.74)</td>
</tr>
<tr>
<td>% Non-perseverative Errors</td>
<td>14.82 (10.81)</td>
<td>16.61 (10.50)</td>
</tr>
<tr>
<td>% Conceptual Level Responses</td>
<td>63.16 (25.36)</td>
<td>52.76 (27.69)</td>
</tr>
<tr>
<td>Categories Completed</td>
<td>4.63 (2.12)</td>
<td>3.63 (2.59)</td>
</tr>
<tr>
<td>Trials to First Category</td>
<td>31.03 (37.34)</td>
<td>38.37 (44.64)</td>
</tr>
<tr>
<td>Failure to Maintain Set</td>
<td>0.63 (1.15)</td>
<td>0.95 (1.16)</td>
</tr>
<tr>
<td>Learning to Learn</td>
<td>0.86 (4.10)</td>
<td>0.15 (6.18)</td>
</tr>
<tr>
<td>(Standard Scores):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Errors</td>
<td>91.00 (17.81)</td>
<td>86.05 (15.86)</td>
</tr>
<tr>
<td>% Perseverative Responses</td>
<td>95.03 (19.50)</td>
<td>89.92 (20.34)</td>
</tr>
<tr>
<td>% Perseverative Errors</td>
<td>94.79 (19.22)</td>
<td>88.74 (19.68)</td>
</tr>
<tr>
<td>% Non-perseverative Errors</td>
<td>90.34 (15.76)</td>
<td>88.18 (16.58)</td>
</tr>
<tr>
<td>% Conceptual Level Responses</td>
<td>90.84 (18.14)</td>
<td>84.84 (16.71)</td>
</tr>
</tbody>
</table>

Table 7.3: Group comparisons of cognitive outcomes.
7.3.1.2 Correlations

The bivariate correlation matrix (Table 7.4) identified a number of significant correlations between participant characteristic variables and cognitive outcomes, which were entered later as control variables in the regression models. Gender, duration since last alcohol and smoker/non-smoker classification were correlated with a small proportion of cognitive measures (gender correlated with immediate verbal memory and reaction time; duration since last consumption of alcohol correlated with immediate and delayed verbal memory and failures to maintain set on the WCST; cigarette smoking correlated with immediate verbal memory), while, as expected, a large number of the outcomes were positively correlated with years of education and negatively correlated with measures of depression and state anxiety. Interestingly, the duration since last consumption of caffeine was positively correlated with scores measuring attention. Thus, an increase in the time since participants had last consumed caffeine was associated with higher scores, an effect inconsistent with the recognised effect of caffeine as a CNS stimulant (Brunyé et al., 2010).

7.3.1.3 Hypothesis testing

The hypotheses predicted an effect of participant group (patient or control) and an interaction effect of group and age, and were tested through a series of individual hierarchical regressions for each of the cognitive outcomes. Significant covariates of the dependent variable were entered in the first block, group (coded $-1/1$) and age (mean-centred) were entered in block 2, and the interaction term (group $\times$ age) was entered in block 3. Statistics for cognitive variables predicted by group or by the interaction term (at levels close to or above the level of statistical significance) are presented in Tables 7.5–7.12. For illustrative purposes, bar charts of the means and standard errors of the four conditions created by the $2 \times 2$ design and interaction charts for estimated marginal means are presented (Figures 7.1–7.8). The estimated marginal mean is the mean of the dependent variable averaged
across all levels of a covariate, i.e., the average of the dependent variable at each of the different levels of the covariate without weighting for the sample size at each level. This means that biases arising from large differences in the \( n \) number at each level are controlled for and the effect of the covariate is thereby minimised. The interaction charts are useful for visualisation of the directionality of effects and for determining the nature of the interaction between variables. It should be noted that the expanded scale on the interaction charts presented herein (compared with that on the bar charts) may exaggerate the magnitude of the differences between groups. The combination of the bar charts and interaction charts is therefore preferable to either presentation alone.

### 7.3.1.4 Intelligence

Years of education, PHQ severity score and SAI total score were entered as controls in block 1. Despite the observed correlations between these variables (Table 7.4), the observed variance inflation factors (VIFs) were less than 10, suggesting the assumption of minimal multicollinearity was not violated. The VIF is an inverse measure of how much of the variability of the specified independent variable is not explained by the other independent variables in the model (Pallant, 2011). Years of education and PHQ severity score significantly contributed to the model (\( \beta = 0.43, \ p < 0.001 \) and \( \beta = -0.30, \ p = 0.03 \), respectively), as did group (\( \beta = 0.33, \ p = 0.04 \)). The positive \( \beta \) coefficient suggests a higher FSIQ in the control group, as patients were coded \(-1\) and controls were coded \(+1\). Neither age nor the interaction term made any significant contribution to the model (see Table 7.5).
Table 7.4: Bivariate correlation matrix of participant characteristics and cognitive outcomes

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>Age</th>
<th>Gender</th>
<th>Years of Education</th>
<th>Smoker</th>
<th>Time since last nicotine</th>
<th>Time since last caffeine</th>
<th>Time since last alcohol</th>
<th>Depression score</th>
<th>State anxiety score</th>
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<tr>
<td>Years of Education</td>
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<td>Time since last alcohol</td>
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<td>State anxiety score</td>
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<td>Estimated FSIQ</td>
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<td>0.28∗</td>
<td>0.28∗</td>
<td>0.25</td>
<td>0.10</td>
<td>-0.25∗</td>
<td>-0.45∗</td>
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<td>0.00</td>
<td>-0.04</td>
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<td>-0.36∗</td>
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<td>-0.07</td>
<td>0.24∗</td>
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<td>0.09</td>
<td>-0.11</td>
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<td>0.10</td>
<td>0.32∗∗</td>
<td>0.13</td>
<td>0.20</td>
<td>-0.05</td>
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<td>-0.29∗</td>
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<tr>
<td>Theme Recall</td>
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<td>-0.15</td>
<td>0.14</td>
<td>0.16</td>
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<td>0.03</td>
<td>-0.30∗</td>
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<td>-0.13</td>
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<td>0.15</td>
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<td>0.21</td>
<td>0.19</td>
<td>-0.01</td>
<td>-0.13</td>
<td>-0.22</td>
<td>-0.23∗</td>
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<td>% Retention</td>
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<td>-0.20</td>
<td>-0.12</td>
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<td>-0.16</td>
<td>0.05</td>
<td>-0.20</td>
<td>-0.03</td>
<td>0.02</td>
<td>-0.10</td>
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<tr>
<td>Forward</td>
<td>0.23∗</td>
<td>-0.12</td>
<td>0.03</td>
<td>0.11</td>
<td>0.17</td>
<td>-0.15</td>
<td>0.17</td>
<td>-0.15</td>
<td>-0.25∗</td>
<td>-0.24∗</td>
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<td>0.23∗</td>
<td>0.01</td>
<td>-0.04</td>
<td>-0.05</td>
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<td>Total</td>
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<td>-0.07</td>
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<td>0.05</td>
<td>-0.21</td>
<td>-0.24∗</td>
<td>-0.22</td>
</tr>
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*p < 0.05, two-tailed, **p < 0.01 two-tailed

Continued on next page …
Table 7.4 (continued from previous page)

<table>
<thead>
<tr>
<th>Attention:</th>
<th>Group</th>
<th>Age</th>
<th>Gender</th>
<th>Years of Education</th>
<th>Smoker</th>
<th>Time since last nicotine</th>
<th>Time since last caffeine</th>
<th>Time since last alcohol</th>
<th>Depression score</th>
<th>State anxiety score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hits</td>
<td>0.17</td>
<td>-0.34**</td>
<td>-0.05</td>
<td>0.24*</td>
<td>-0.02</td>
<td>0.08</td>
<td>0.11</td>
<td>0.01</td>
<td>-0.31**</td>
<td>-0.21</td>
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<tr>
<td>False alarms</td>
<td>-0.17</td>
<td>0.35**</td>
<td>-0.04</td>
<td>-0.34**</td>
<td>0.07</td>
<td>-0.33</td>
<td>-0.30*</td>
<td>0.12</td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td>Randoms</td>
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<td>0.35**</td>
<td>0.03</td>
<td>-0.36**</td>
<td>0.01</td>
<td>-0.17</td>
<td>-0.16</td>
<td>0.06</td>
<td>0.14</td>
<td>0.18</td>
</tr>
<tr>
<td>D-Prime</td>
<td>0.23*</td>
<td>-0.45**</td>
<td>0.00</td>
<td>0.41**</td>
<td>-0.06</td>
<td>0.31</td>
<td>0.24*</td>
<td>-0.10</td>
<td>-0.28*</td>
<td>-0.16</td>
</tr>
<tr>
<td>T-score</td>
<td>0.22</td>
<td>-0.44**</td>
<td>-0.01</td>
<td>0.39**</td>
<td>-0.08</td>
<td>0.36</td>
<td>0.25*</td>
<td>-0.09</td>
<td>-0.29*</td>
<td>-0.23*</td>
</tr>
<tr>
<td>T-score corrected</td>
<td>0.18</td>
<td>-0.29*</td>
<td>0.13</td>
<td>0.35**</td>
<td>-0.08</td>
<td>0.27</td>
<td>0.19</td>
<td>0.00</td>
<td>-0.30*</td>
<td>-0.27*</td>
</tr>
</tbody>
</table>

**Psychomotor Speed:**

| Hit reaction time         | 0.11  | 0.15    | 0.25*  | -0.09              | 0.13   | 0.12                     | -0.02                    | -0.07                  | -0.07            | 0.04                |
| False Alarm reaction time | 0.01  | 0.02    | 0.23*  | 0.08               | 0.11   | 0.25                     | 0.05                     | -0.07                  | 0.09             | 0.14                |

**Executive Function:**

| Errors                    | 0.15  | -0.17   | 0.11   | -0.04              | -0.02  | 0.11                     | 0.24*                    | -0.16                  | -0.13            | -0.11               |
| Perseverative responses   | 0.13  | -0.07   | 0.17   | -0.20              | -0.03  | 0.13                     | 0.24*                    | -0.13                  | -0.15            | -0.01               |
| Perseverative errors      | 0.16  | -0.08   | 0.17   | -0.18              | -0.03  | 0.12                     | 0.23                     | -0.15                  | -0.16            | -0.03               |
| Non-perseverative errors  | 0.07  | -0.24*  | 0.07   | 0.03               | -0.01  | 0.06                     | 0.20                     | -0.10                  | -0.04            | -0.17               |
| Conceptual level responses| 0.18  | -0.19   | 0.09   | -0.01              | 0.02   | 0.06                     | 0.24*                    | -0.16                  | -0.14            | -0.12               |
| Categories completed      | 0.20  | -0.29*  | -0.01  | 0.26*              | 0.00   | 0.07                     | 0.19                     | -0.18                  | -0.24*           | -0.21               |
| Trials to 1st category    | -0.14 | 0.17    | -0.09  | -0.15              | 0.06   | 0.15                     | -0.27*                   | -0.00                  | 0.19             | 0.27*               |
| Failure to maintain set   | -0.20 | 0.19    | 0.11   | -0.06              | -0.10  | 0.26                     | -0.15                    | 0.35**                 | 0.27*            | 0.13                |
| Learning to learn         | 0.06  | -0.19   | 0.09   | -0.03              | -0.09  | 0.21                     | -0.16                    | -0.11                  | 0.05             | 0.13                |

*p < 0.05, two-tailed, **p < 0.01 two-tailed

Table 7.4: Bivariate correlation matrix of participant characteristics and cognitive outcomes.
Table 7.5: Hierarchical multiple regression predicting full-scale IQ.

<table>
<thead>
<tr>
<th>Block</th>
<th>$\beta$</th>
<th>SE</th>
<th>$p$</th>
<th>$\Delta R^2$</th>
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<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>0.387**</td>
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<tr>
<td>Years of Education</td>
<td>0.425</td>
<td>0.455</td>
<td>&lt; 0.001</td>
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</tr>
<tr>
<td>PHQ severity score</td>
<td>−0.296</td>
<td>0.295</td>
<td>0.025</td>
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</tr>
<tr>
<td>SAI total score</td>
<td>−0.075</td>
<td>0.170</td>
<td>0.575</td>
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</tr>
<tr>
<td>2</td>
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<td></td>
<td></td>
<td>0.064*</td>
</tr>
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<td>Years of Education</td>
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<td>0.462</td>
<td>&lt; 0.001</td>
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<tr>
<td>PHQ severity score</td>
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<td>0.978</td>
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</tr>
<tr>
<td>SAI total score</td>
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<tr>
<td>Group</td>
<td>0.334</td>
<td>2.529</td>
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<td>Age</td>
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<td>0.149</td>
<td>0.108</td>
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<tr>
<td>3</td>
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<tr>
<td>Years of Education</td>
<td>0.370</td>
<td>0.465</td>
<td>&lt; 0.001</td>
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<tr>
<td>PHQ severity score</td>
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<td>Group</td>
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<td>Group * Age</td>
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<td>Total $R^2$</td>
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Figure 7.1: (a) Mean and standard errors for estimated FSIQ for old (age ≥ 45 years) and young (age < 45) chronic pain patients and controls. (b) Interaction chart for estimated marginal means for FSIQ for old and young patients and controls.
7.3.1.5 Immediate verbal memory

Immediate verbal memory, assessed using the Logical Memory I test of the WMS-III (Wechsler, 1997c), was associated with story-unit and theme-unit recall scores and a learning slope score. Group, age, and their interaction term made no contribution to the story or theme unit regression models. There were no significant predictor variables of the theme unit model and only gender significantly contributed to the story recall unit model ($\beta = 2.115, p = 0.039$). In the case of learning slope, the contribution of SAI total score (entered in the first block) approached significance ($\beta = -0.270, p = 0.062$). There was a significant contribution of age ($\beta = -0.288, p = 0.015$); the interaction term was also associated with an effect close to statistical significance ($\beta = 0.198, p = 0.075$, Table 7.6) indicating that older pain patients performed worse than controls of a similar age and worse than younger pain patients.

7.3.1.6 Delayed verbal memory

Delayed verbal memory, assessed using the Logical Memory II test of the WMS-III (Wechsler, 1997c), was also associated with story-unit and theme-unit recall scores, as well as with a recognition-memory index and with percentage retention. Similar to immediate verbal memory, neither group, age, nor their interaction significantly contributed to either story or theme unit models and none of the additional predictor variables significantly affected these models. In addition, no significant predictors of percentage retention were identified (though the effect of age was close to significance: $\beta = -0.259, p = 0.079$). Recognition memory, however, was significantly predicted by both age ($\beta = -0.273, p = 0.013$) and the age-group interaction term ($\beta = 0.304, p = 0.006$, Table 7.7), with poor performance particularly in older pain patients.
Table 7.6: Hierarchical multiple regression predicting verbal learning slope.

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<tr>
<th>Block</th>
<th>β</th>
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<th>p</th>
<th>ΔR^2</th>
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<tr>
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<td>0.075</td>
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<tr>
<td>Total R^2</td>
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<td></td>
<td>0.186</td>
</tr>
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</table>

Figure 7.2: (a) Mean and standard errors for verbal learning slope for old (age ≥ 45 years) and young (age < 45) chronic pain patients and for controls. (b) Interaction chart for estimated marginal means for verbal learning slope memory for old and young patients and controls.
Table 7.7: Hierarchical multiple regression predicting verbal recognition memory.

<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>SE</th>
<th>p</th>
<th>ΔR²</th>
</tr>
</thead>
<tbody>
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<td><strong>Block 1</strong></td>
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<td></td>
<td></td>
<td>0.044</td>
</tr>
<tr>
<td>SAI total score</td>
<td>−0.210</td>
<td>0.026</td>
<td>0.069</td>
<td></td>
</tr>
<tr>
<td><strong>Block 2</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.079*</td>
</tr>
<tr>
<td>SAI total score</td>
<td>−0.162</td>
<td>0.031</td>
<td>0.240</td>
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</tr>
<tr>
<td>Group</td>
<td>0.116</td>
<td>0.380</td>
<td>0.396</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.252</td>
<td>0.031</td>
<td>0.028</td>
<td></td>
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<tr>
<td><strong>Block 3</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.090**</td>
</tr>
<tr>
<td>SAI total score</td>
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<td>0.030</td>
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</tr>
<tr>
<td>Group</td>
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<td>0.364</td>
<td>0.544</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.273</td>
<td>0.030</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Group * Age</td>
<td>0.304</td>
<td>0.030</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td><strong>Total R²</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.212</td>
</tr>
</tbody>
</table>

Figure 7.3: (a) Mean and standard errors for verbal recognition memory for old (age ≥ 45 years) and young (age < 45) chronic pain patients and controls. (b) Interaction chart for estimated marginal means for verbal recognition memory for old and young patients and controls.
Table 7.8: Hierarchical multiple regression predicting spatial span reversal.

<table>
<thead>
<tr>
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<tr>
<td>Years of Education</td>
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<td>0.042</td>
<td>0.054*</td>
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<td>Block 2</td>
<td></td>
<td></td>
<td></td>
<td>0.060</td>
</tr>
<tr>
<td>Years of Education</td>
<td>0.231</td>
<td>0.114</td>
<td>0.053</td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>0.208</td>
<td>0.372</td>
<td>0.071</td>
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</tr>
<tr>
<td>Age</td>
<td>0.144</td>
<td>0.038</td>
<td>0.217</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Years of Education</td>
<td>0.231</td>
<td>0.114</td>
<td>0.055</td>
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</tr>
<tr>
<td>Group</td>
<td>0.208</td>
<td>0.374</td>
<td>0.073</td>
<td></td>
</tr>
<tr>
<td>Age</td>
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<td>0.038</td>
<td>0.222</td>
<td></td>
</tr>
<tr>
<td>Group * Age</td>
<td>0.011</td>
<td>0.037</td>
<td>0.920</td>
<td></td>
</tr>
<tr>
<td>Total R²</td>
<td></td>
<td></td>
<td></td>
<td>0.114</td>
</tr>
</tbody>
</table>

Figure 7.4: (a) Mean and standard errors for spatial span reverse scaled score for old (age ≥ 45 years) and young (age < 45) chronic pain patients and controls. (b) Interaction chart for estimated marginal means for spatial span reverse scaled score for old and young patients and controls.
7.3.1.7 Spatial memory

Spatial memory was assessed in forward and reverse trial of the spatial span task. Total spatial span and forward trial scores were not predicted by any of the three main independent variables of interest (group, age, group by age interaction), or by any of the control measures entered into the model. Effects close to the level of statistical significance were associated with group ($\beta = 0.208$, $p = 0.071$) and years of education ($\beta = 0.231$, $p = 0.053$) in the reverse scores model (Table 7.8).

7.3.1.8 Attention

Attention was measured using the CPT, the outcome variables of which were the numbers of hits, false alarms and random responses. The number of hits in the CPT was significantly predicted by the main independent variable of age ($\beta = -0.354$, $p = 0.002$) and there was also a trend for a contribution of group ($\beta = -0.358$, $p = 0.057$, Table 7.9). The negative association in this case suggests a reduced number of hits in the control group compared with the patient group. The control variable of depressive symptom score (PHQ severity) was also a significant contributor to the model ($\beta = -0.592$, $p = 0.002$). Age ($\beta = 0.283$, $p = 0.015$) and the age by group interaction ($\beta = -0.252$, $p = 0.018$) term made significant contributions to the regression model for number of false alarms (Table 7.10). The number of random responses was influenced by age ($\beta = 0.355$, $p = 0.001$) and there was a near-significant effect for the age by group interaction term ($\beta = 0.200$, $p = 0.057$, Table 7.11). For the reasons described in Section 7.2.3, separate regression models were fitted for raw D-prime scores, and both uncorrected and corrected T-scores. The results were similar for the three models, with age (raw D-prime: $\beta = -0.344$, $p = 0.003$; uncorrected T-score: $\beta = -0.271$, $p = 0.022$; corrected T-score: $\beta = -0.254$, $p = 0.037$) and PHQ severity scores (raw D-prime: $\beta = -0.334$, $p = 0.073$; uncorrected T-score: $\beta = -0.391$, $p = 0.044$; corrected T-score: $\beta = -0.552$, $p = 0.018$) emerging as predictors for all three dependent variables. There were no significant effects of group or interaction.
Table 7.9: Hierarchical multiple regression predicting the number of hit responses in the CPT.

<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>SE</th>
<th>p</th>
<th>ΔR²</th>
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<td></td>
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<tr>
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<td>0.131</td>
<td>0.135</td>
<td>0.123**</td>
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<td>PHQ severity score</td>
<td>-0.268</td>
<td>0.064</td>
<td>0.020</td>
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<td>Block 2</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Years of Education</td>
<td>0.066</td>
<td>0.127</td>
<td>0.548</td>
<td>0.137**</td>
</tr>
<tr>
<td>PHQ severity score</td>
<td>-0.595</td>
<td>0.106</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>-0.361</td>
<td>0.726</td>
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<td></td>
</tr>
<tr>
<td>Age</td>
<td>-0.356</td>
<td>0.042</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Block 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Years of Education</td>
<td>0.067</td>
<td>0.128</td>
<td>0.549</td>
<td>0.002</td>
</tr>
<tr>
<td>PHQ severity score</td>
<td>-0.592</td>
<td>0.106</td>
<td>0.002</td>
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</tr>
<tr>
<td>Group</td>
<td>-0.358</td>
<td>0.730</td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>-0.354</td>
<td>0.042</td>
<td>0.002</td>
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<td>Group * Age</td>
<td>-0.040</td>
<td>0.040</td>
<td>0.701</td>
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</tr>
<tr>
<td>Total R²</td>
<td></td>
<td></td>
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<td>0.262</td>
</tr>
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</table>

Figure 7.5: (a) Mean and standard errors for CPT hit responses for old (age ≥ 45 years) and young (age < 45) chronic pain patients and controls. (b) Interaction chart for estimated marginal means for CPT hit responses for old and young patients and controls.
Table 7.10: Hierarchical multiple regression predicting the number of false alarm responses in the CPT.

<table>
<thead>
<tr>
<th>Block</th>
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<th>ΔR²</th>
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</thead>
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<td>0.149</td>
<td>0.006</td>
<td>0.151**</td>
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<td>−0.315</td>
<td>0.149</td>
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<td>Last caffeine</td>
<td>−0.202</td>
<td>0.001</td>
<td>0.074</td>
<td></td>
</tr>
<tr>
<td>Block 2</td>
<td>0.080*</td>
<td>0.147</td>
<td>0.001</td>
<td>0.080*</td>
</tr>
<tr>
<td>Years of Education</td>
<td>−0.219</td>
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</tr>
<tr>
<td>Last caffeine</td>
<td>−0.115</td>
<td>0.001</td>
<td>0.315</td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>−0.144</td>
<td>0.499</td>
<td>0.192</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.269</td>
<td>0.053</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>Block 3</td>
<td>0.063*</td>
<td>0.147</td>
<td>0.001</td>
<td>0.063*</td>
</tr>
<tr>
<td>Years of Education</td>
<td>−0.219</td>
<td>0.147</td>
<td>0.050</td>
<td></td>
</tr>
<tr>
<td>Last caffeine</td>
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</tr>
<tr>
<td>Group</td>
<td>−0.144</td>
<td>0.482</td>
<td>0.178</td>
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</tr>
<tr>
<td>Age</td>
<td>0.283</td>
<td>0.051</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>Group * Age</td>
<td>−0.252</td>
<td>0.047</td>
<td>0.018</td>
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</tr>
<tr>
<td>Total R²</td>
<td>0.294</td>
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</table>

Figure 7.6: Mean and standard errors for CPT false alarm responses for old (age ≥ 45 years) and young (age < 45) chronic pain patients and controls. (b) Interaction chart for estimated marginal means for CPT false alarm responses for old and young patients and controls.
Table 7.11: Hierarchical multiple regression predicting the number of random responses in the CPT.

Figure 7.7: (a) Mean and standard errors for CPT random responses for old (age ≥ 45 years) and young (age < 45) chronic pain patients and controls. (b) Interaction chart for estimated marginal means for CPT random responses for old and young patients and controls.
7.3.1.9 Psychomotor speed

The reaction times to both hits and false alarms in the CPT task were considered measures of psychomotor speed. There were no main effects of age or group. Though the individual contributions of age and group were non-significant, the group $\times$ age interaction term significantly contributed to the model for “hit” reaction time ($\beta = 0.240, p = 0.033$, Table 7.12). This interaction effect was approaching significance in the case of “false alarm” reaction time ($\beta = 0.209, p = 0.076$), though the overall model was not significant.

7.3.1.10 Executive function

The independent variables of group, age, and age by group interaction did not predict any of the WCST standard scores or the secondary outcomes (number of categories completed, number of trials to first category, failure to maintain set and learning-to-learn score). The only significant predictor was age (number of categories completed: $\beta = -0.281, p = 0.016$), whereby older people achieved lower scores than younger participants.

<table>
<thead>
<tr>
<th></th>
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<th>SE</th>
<th>$p$</th>
<th>$\Delta R^2$</th>
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</thead>
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<td></td>
<td></td>
<td>0.062*</td>
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<td>Gender</td>
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<td>15.968</td>
<td>0.030</td>
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<td></td>
<td></td>
<td>0.037</td>
</tr>
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<td>Gender</td>
<td>0.250</td>
<td>15.865</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>0.120</td>
<td>7.852</td>
<td>0.289</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.160</td>
<td>0.779</td>
<td>0.159</td>
<td></td>
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<tr>
<td><strong>Block 3</strong></td>
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<td></td>
<td></td>
<td>0.056*</td>
</tr>
<tr>
<td>Gender</td>
<td>0.212</td>
<td>15.661</td>
<td>0.058</td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>0.119</td>
<td>7.656</td>
<td>0.280</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.148</td>
<td>0.761</td>
<td>0.180</td>
<td></td>
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<tr>
<td>Group $\times$ Age</td>
<td>0.240</td>
<td>0.770</td>
<td>0.033</td>
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</tr>
<tr>
<td><strong>Total $R^2$</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.155</td>
</tr>
</tbody>
</table>

Table 7.12: Hierarchical multiple regression predicting hit reaction time in the CPT.
Figure 7.8: (a) Mean and standard errors for CPT hit response reaction time for old (age $\geq 45$ years) and young (age $< 45$) chronic pain patients and controls. (b) Interaction chart for estimated marginal means for CPT hit response reaction time for old and young patients and controls.

7.3.2 Phase II: Analysis by pain variable

7.3.2.1 Descriptive statistics

Table 7.13 presents descriptive statistics for variables unique to the pain patient group and Table 7.14 provides an overview of patients’ medication status. Seventy-nine per cent of patients were receiving long-term medication for the treatment of pain. Opioids were the most commonly prescribed class of medication, followed by anticonvulsants. Only pain medications were included in the classification of patient medication. The “other” category included classes of analgesics taken by a small number of participants within the sample. These treatments included: antispasmodics, benzodiazepines and other sedative hypnotics, local anaesthetics, and TRPV1 ligands (capsaicin), as well as adjunctive therapies such as acupuncture and spinal cord stimulation. In cases where a drug had more than one indication (for example, benzodiazepines used as a muscle relaxant, as an anxiolytic or as treatment for insomnia), the precise reason for which it was prescribed was not queried. There was no correlation between the number of additional medications and any of the cognitive outcomes (Table 7.16). More than half (55%) of the pa-
Table 7.12: Pain variables descriptives.

<table>
<thead>
<tr>
<th>Pain Variable</th>
<th>Mean (SD)</th>
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<tbody>
<tr>
<td>Present pain intensity</td>
<td>5.63 (3.63)</td>
</tr>
<tr>
<td>Average pain intensity</td>
<td>73.16 (13.67)</td>
</tr>
<tr>
<td>Pain-related disability score</td>
<td>79.39 (15.12)</td>
</tr>
<tr>
<td>Chronic pain grade</td>
<td>3.53 (0.65)</td>
</tr>
<tr>
<td>Number of painful areas</td>
<td>5.87 (3.63)</td>
</tr>
<tr>
<td>Pain chronicity (months)</td>
<td>101.4 (86.4)</td>
</tr>
<tr>
<td>Self-assessed effect of pain on cognition</td>
<td>6.45 (2.74)</td>
</tr>
</tbody>
</table>

Table 7.13: Pain variables descriptives.

<table>
<thead>
<tr>
<th>Medication variable</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>% receiving medication</td>
<td>78.9</td>
</tr>
<tr>
<td>% receiving opioids</td>
<td>63.2</td>
</tr>
<tr>
<td>% receiving anticonvulsants</td>
<td>42.1</td>
</tr>
<tr>
<td>% receiving antidepressants</td>
<td>31.6</td>
</tr>
<tr>
<td>% receiving NSAIDs</td>
<td>39.5</td>
</tr>
<tr>
<td>% receiving other medication</td>
<td>39.5</td>
</tr>
<tr>
<td>% pain relief from medications</td>
<td>36.59 (27.04)</td>
</tr>
<tr>
<td>Total number of medications</td>
<td>2.8 (2.0)</td>
</tr>
</tbody>
</table>

Table 7.14: Breakdown of patient medications.

Patient participants were taking three or more different drugs to manage their pain. The average subjectively-reported relief provided by pharmacological treatments was 37%.

7.3.2.2 Correlations

A correlation matrix with variables identical to those in Table 7.4, but using data from pain participants only, was used to identify general patient characteristics that correlated with cognitive outcomes.

Similar to Phase I, years of education correlated with a large proportion of the outcome measures. The only other significant correlations were between: duration since last consumption of nicotine and the spatial span forward trial; duration since last consumption of alcohol and both hit reaction time and WCST failures to maintain set; depression score and number of hits, false alarm reaction time
and WCST failures to maintain set; and SAI total score and false alarm reaction time (see Table 7.15).

A further correlation matrix (Table 7.16) was used to identify correlations between cognitive outcomes and patient-specific variables, including medications and patient’s subjective assessment of the effect of their pain on cognitive function. Anticonvulsant treatment was found to significantly correlate with learning slope performance ($r_{pb} = 0.368$, $p < 0.05$) suggesting lower scores in those receiving anticonvulsant medications. On the other hand, antidepressant treatment was associated with higher scores on the attention task ($r_{pb} = -0.376$, $p < 0.05$ D-prime, $r_{pb} = -0.348$, $p < 0.05$ T-score corrected) and improved WCST performance as indicated by a decrease in the number of trials required to complete the first category ($r_{pb} = 0.338$, $p < 0.05$). Self-assessment of the effect of pain on cognitive function was positively correlated with the failures to maintain set in the WCST ($r_{pb} = 0.448$, $p < 0.01$). All significant correlations were entered as control variables in the hierarchical regression analyses.
Table 7.15: Correlation matrix of general *patient* characteristics and cognitive outcomes

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Gender</th>
<th>Years of Education</th>
<th>Smoker</th>
<th>Time since last nicotine</th>
<th>Time since last caffeine</th>
<th>Time since last alcohol</th>
<th>Depression score</th>
<th>State anxiety score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated FSIQ</td>
<td>−0.27</td>
<td>0.08</td>
<td>0.53***</td>
<td>0.08</td>
<td>0.17</td>
<td>0.02</td>
<td>−0.24</td>
<td>−0.08</td>
<td>−0.05</td>
</tr>
<tr>
<td><strong>Immediate Verbal</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Memory:</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unit Recall</td>
<td>−0.32</td>
<td>0.31</td>
<td>0.43***</td>
<td>0.03</td>
<td>0.06</td>
<td>0.15</td>
<td>−0.08</td>
<td>−0.06</td>
<td>−0.02</td>
</tr>
<tr>
<td>Theme Recall</td>
<td>−0.13</td>
<td>0.26</td>
<td>0.33*</td>
<td>0.17</td>
<td>−0.07</td>
<td>−0.00</td>
<td>−0.03</td>
<td>0.03</td>
<td>0.14</td>
</tr>
<tr>
<td>Learning slope</td>
<td>−0.43**</td>
<td>0.15</td>
<td>0.23</td>
<td>0.03</td>
<td>0.11</td>
<td>0.30</td>
<td>−0.13</td>
<td>−0.04</td>
<td>−0.22</td>
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<tr>
<td><strong>Delayed Verbal Memory:</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unit Recall</td>
<td>−0.26</td>
<td>0.12</td>
<td>0.45***</td>
<td>−0.03</td>
<td>0.01</td>
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Table 7.15: Correlation matrix of general patient characteristics and cognitive outcomes.

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<th>Time since last caffeine</th>
<th>Time since last alcohol</th>
<th>Depression score</th>
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Table 7.16: Correlation matrix of patient-specific characteristics and cognitive outcomes

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<th>Anti-depressant</th>
<th>NSAID</th>
<th>Other</th>
<th>Number of medications</th>
<th>% Pain relief</th>
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Table 7.16:  continued from previous page.

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<th>Other</th>
<th>Number of medications</th>
<th>% Pain relief</th>
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</table>

Table 7.16: Correlation matrix of patient-specific characteristics and cognitive outcomes.
7.3.2.3 Hypothesis testing

The second set of hypotheses stated that the pain variables, and the interaction of these variables with age, would predict differences in cognitive outcomes. Hypotheses were again tested through a series of hierarchical regressions. Significant covariates of the dependent variable were entered in the first block. Each pain variable (present pain intensity, average pain intensity, pain-related disability score, pain chronicity and the number of painful areas) was tested individually. Both the pain variable and age were centred and entered in block 2, and the interaction term was entered in block 3. Statistics for cognitive variables predicted by pain variables or by the interaction term are presented in Tables 7.17–7.28.

Chronicity significantly contributed to the models of FSIQ ($\beta = 0.314$, $p \leq 0.05$, Table 7.17), immediate verbal memory ($\beta = 0.474$, $p = 0.003$, Table 7.18) and delayed verbal memory ($\beta = 0.402$, $p = 0.016$, Table 7.19). The contribution of chronicity was also nearing significance in the hit response reaction time model ($\beta = 0.356$, $p = 0.07$, Table 7.20). The association was positive in all cases suggesting that performance improved with an increasing duration of pain.

<table>
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<th>$\beta$</th>
<th>SE</th>
<th>$p$</th>
<th>$\Delta R^2$</th>
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<td>0.28**</td>
</tr>
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<tr>
<td>Pain chronicity</td>
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<td>0.02</td>
<td>0.05</td>
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<tr>
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<td>0.22</td>
<td>0.13</td>
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</tr>
<tr>
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<td>0.56</td>
<td>0.00</td>
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<tr>
<td>Pain chronicity</td>
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<td>0.03</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Age</td>
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<td>0.23</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Pain chronicity * Age</td>
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<td>Total $R^2$</td>
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Table 7.17: Hierarchical multiple regression predicting full-scale IQ (FSIQ) with pain chronicity as an independent variable.
The number of painful areas was found to be an effective predictor of immediate verbal memory (story unit recall: $\beta = -0.341$, $p = 0.049$, Table 7.21; theme unit recall: $\beta = -0.372$, $p = 0.038$) and delayed verbal memory ($\beta = -0.351$, $p = 0.035$, Table 7.22), whereby “more painful sites” was associated with worse performance. The interaction of the number of painful areas and age was a significant contributor to the model of the spatial span reverse trial ($\beta = -0.361$, $p = 0.025$, ANOVA $p = 0.058$, Table 7.23) and the number of random responses on the CPT ($\beta = 0.304$, $p = 0.034$, Table 7.24).

Chronic pain grade significantly predicted D-prime scores of attention ($\beta = -0.430$, $p = 0.002$, Table 7.25), as did disability score ($\beta = -0.281$, $p = 0.031$, Table 7.26). The interaction term of disability score by age also made a significant contribution to the memory retention model ($\beta = -0.473$, $p = 0.006$, Table 7.27), while the interaction of present pain intensity and age had an effect close to significant on the number of hits in the CPT ($\beta = -0.31$, $p = 0.066$, Table 7.28). The sign of the $\beta$ values indicates that worse pain was associated with lower cognitive performance. In general, therefore, aspects of verbal memory and attention appear to be related to a variety of pain measurements. No main effects were observed on any of the cognitive outcome measures due to total number of medications, or due to treatment with any of the medication classifications.
### Table 7.19: Hierarchical multiple regression predicting Logical Memory II story unit recall with pain chronicity as an independent variable.

<table>
<thead>
<tr>
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<th>( \Delta R^2 )</th>
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<td>Pain chronicity</td>
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<td>Age</td>
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<th>( \Delta R^2 )</th>
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<tr>
<td>Pain chronicity</td>
<td>0.40</td>
<td>0.01</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.29</td>
<td>0.04</td>
<td>0.08</td>
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</tr>
<tr>
<td>Pain chronicity * Age</td>
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<td>0.00</td>
<td>0.49</td>
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| Total \( R^2 \)    |       |     |      | 0.36             |

### Table 7.20: Hierarchical multiple regression predicting hit response reaction time with pain chronicity as an independent variable.

<table>
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<th>p</th>
<th>( \Delta R^2 )</th>
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<td>Last Alcohol</td>
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<th>SE</th>
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<th>( \Delta R^2 )</th>
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<td>0.00</td>
<td>0.23*</td>
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<td>Pain chronicity</td>
<td>0.33</td>
<td>0.14</td>
<td>0.06</td>
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<tr>
<td>Age</td>
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<td>1.47</td>
<td>0.01</td>
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<th>SE</th>
<th>p</th>
<th>( \Delta R^2 )</th>
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</thead>
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<td>0.07</td>
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<td>Age</td>
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<td>1.67</td>
<td>0.02</td>
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<tr>
<td>Pain chronicity * Age</td>
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<td>0.02</td>
<td>0.75</td>
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<p>| Total ( R^2 )    |       |     |      | 0.36             |</p>
<table>
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<th>p</th>
<th>ΔR²</th>
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<th>p</th>
<th>ΔR²</th>
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<td>0.13</td>
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<tr>
<td># of painful areas</td>
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<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.17</td>
<td>0.05</td>
<td>0.26</td>
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</tbody>
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<table>
<thead>
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<th>p</th>
<th>ΔR²</th>
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<tr>
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<td>0.53</td>
<td>0.14</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td># of painful areas</td>
<td>−0.34</td>
<td>0.14</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.17</td>
<td>0.05</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td># of painful areas * Age</td>
<td>−0.04</td>
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Total $R^2$ = 0.33

Table 7.21: Hierarchical multiple regression predicting Logical Memory I story unit recall with number of painful areas as an independent variable.

<table>
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<th>ΔR²</th>
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<tr>
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<td>0.00</td>
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<table>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.11</td>
<td>0.00</td>
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</tr>
<tr>
<td># of painful areas</td>
<td>−0.36</td>
<td>0.11</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.09</td>
<td>0.04</td>
<td>0.53</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Block 3</th>
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<th>p</th>
<th>ΔR²</th>
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<tbody>
<tr>
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<tr>
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<td>0.01</td>
</tr>
<tr>
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<td>0.11</td>
<td>0.04</td>
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<tr>
<td>Age</td>
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<td>0.04</td>
<td>0.52</td>
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</tr>
<tr>
<td># of painful areas * Age</td>
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Total $R^2$ = 0.35

Table 7.22: Hierarchical multiple regression predicting Logical Memory II story unit recall with number of painful areas as an independent variable.
### Table 7.23: Hierarchical multiple regression predicting spatial span reversal with number of painful areas as an independent variable.

<table>
<thead>
<tr>
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<th>SE</th>
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<th>ΔR²</th>
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<tbody>
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<td></td>
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<td>0.07</td>
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<tr>
<td># of painful areas</td>
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<td>0.16</td>
<td>0.14</td>
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</tr>
<tr>
<td>Age</td>
<td>0.05</td>
<td>0.06</td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

|          |       |      |       | 0.13*|
| Block 2  |       |      |       |      |
| # of painful areas | 0.27  | 0.15 | 0.10  |      |
| Age      | 0.05  | 0.06 | 0.73  |      |
| # of painful areas * Age | −0.36 | 0.02 | 0.03  |      |

|          |       |      |       | 0.20 |
| Block 3  |       |      |       |      |
| Years of Education | −0.15 | 0.17 | 0.36  |      |
| # of painful areas | −0.01 | 0.17 | 0.95  |      |
| Age      | 0.47  | 0.06 | 0.00  |      |
| # of painful areas * Age | 0.30  | 0.02 | 0.03  |      |

|          |       |      |       | 0.39 |

### Table 7.24: Hierarchical multiple regression predicting the number of random responses on the CPT with number of painful areas as an independent variable.

<table>
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<tr>
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<th>β</th>
<th>SE</th>
<th>p</th>
<th>ΔR²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block 1</td>
<td></td>
<td></td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>Years of Education</td>
<td>−0.30</td>
<td>0.15</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

|          |       |      |       | 0.21*|
| Block 2  |       |      |       |      |
| Years of Education | −0.20 | 0.18 | 0.24  |      |
| # of painful areas | 0.03  | 0.18 | 0.87  |      |
| Age      | 0.46  | 0.06 | 0.00  |      |

|          |       |      |       | 0.09*|
| Block 3  |       |      |       |      |
| Years of Education | −0.15 | 0.17 | 0.36  |      |
| # of painful areas | −0.01 | 0.17 | 0.95  |      |
| Age      | 0.47  | 0.06 | 0.00  |      |
| # of painful areas * Age | 0.30  | 0.02 | 0.03  |      |

|          |       |      |       | 0.39 |

297
### Table 7.25: Hierarchical multiple regression predicting CPT D-prime score with Chronic Pain Grade as an independent variable.

<table>
<thead>
<tr>
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<th>SE</th>
<th>p</th>
<th>ΔR²</th>
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</thead>
<tbody>
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<td></td>
<td>0.28**</td>
</tr>
<tr>
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</tr>
<tr>
<td>Antidepressant treatment</td>
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<td>0.26</td>
<td>0.03</td>
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</tr>
<tr>
<td>Block 2</td>
<td></td>
<td></td>
<td></td>
<td>0.29**</td>
</tr>
<tr>
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<td>0.03</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Antidepressant treatment</td>
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<td>0.22</td>
<td>0.00</td>
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</tr>
<tr>
<td>Chronic pain grade</td>
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<td>0.16</td>
<td>0.00</td>
<td></td>
</tr>
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<td>&lt;0.001</td>
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<tr>
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<td></td>
<td></td>
<td>0.02</td>
</tr>
<tr>
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<td>0.03</td>
<td>0.03</td>
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</tr>
<tr>
<td>Antidepressant treatment</td>
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<td>0.22</td>
<td>0.00</td>
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</tr>
<tr>
<td>Chronic pain grade</td>
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<td>0.16</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>-0.55</td>
<td>0.01</td>
<td>&lt;0.001</td>
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<tr>
<td>Chronic pain grade * Age</td>
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Table 7.26: Hierarchical multiple regression predicting CPT D-prime score with pain-related disability as an independent variable.

<table>
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<th>p</th>
<th>ΔR²</th>
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<td></td>
<td></td>
<td></td>
<td>0.28**</td>
</tr>
<tr>
<td>Years of Education</td>
<td>0.37</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Antidepressant treatment</td>
<td>-0.34</td>
<td>0.26</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Block 2</td>
<td></td>
<td></td>
<td></td>
<td>0.23**</td>
</tr>
<tr>
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<td>0.24</td>
<td>0.03</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Antidepressant treatment</td>
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<td>0.22</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Disability score</td>
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<td>0.01</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
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<td>0.01</td>
<td>0.00</td>
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</tr>
<tr>
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<td>0.03</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Antidepressant treatment</td>
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</tr>
<tr>
<td>Disability score</td>
<td>-0.28</td>
<td>0.01</td>
<td>0.03</td>
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</tr>
<tr>
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<td>0.01</td>
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<td>0.00</td>
<td>1.00</td>
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</tr>
<tr>
<td>Total R²</td>
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### Table 7.27: Hierarchical multiple regression predicting verbal memory retention with pain-related disability as an independent variable.

<table>
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<th>ΔR²</th>
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<td>0.06</td>
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<tr>
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<td>0.13</td>
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</tr>
<tr>
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<td>−0.07</td>
<td>0.22</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>Disability score * Age</td>
<td>−0.47</td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Total R²</td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
</tbody>
</table>

Table 7.27: Hierarchical multiple regression predicting verbal memory retention with pain-related disability as an independent variable.

### Table 7.28: Hierarchical multiple regression predicting the number of hit responses on the CPT with present pain intensity as an independent variable.

<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>SE</th>
<th>p</th>
<th>ΔR²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block 1</td>
<td></td>
<td></td>
<td></td>
<td>0.131*</td>
</tr>
<tr>
<td>PHQ severity score</td>
<td>−0.36</td>
<td>0.12</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Block 2</td>
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<td></td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>PHQ severity score</td>
<td>−0.39</td>
<td>0.12</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Present pain intensity</td>
<td>−0.08</td>
<td>0.30</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.31</td>
<td>0.06</td>
<td>0.05</td>
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<tr>
<td>Block 3</td>
<td></td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>PHQ severity score</td>
<td>−0.47</td>
<td>0.12</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Present pain intensity</td>
<td>0.00</td>
<td>0.30</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.22</td>
<td>0.06</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Present pain intensity * Age</td>
<td>−0.31</td>
<td>0.03</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Total R²</td>
<td></td>
<td></td>
<td></td>
<td>0.31</td>
</tr>
</tbody>
</table>

Table 7.28: Hierarchical multiple regression predicting the number of hit responses on the CPT with present pain intensity as an independent variable.
7.4 Discussion

This experiment provides further evidence for significant impairments of performance on tasks requiring memory and attention in chronic pain patients. IQ was significantly lower in patients than in matched controls. There was a trend for pain-related deficits in reversal of spatial memory in the spatial span task, and the age×pain interaction term negatively predicted verbal memory performance. Though attention did not appear to be associated with pain, or the interaction between age and pain, a pattern of abnormal responding on the attention task was observed in pain patients, particularly in the older group. Executive function was not predicted by the variables of interest (pain or pain×age interaction). Individual pain variables, including the number of painful areas, chronic pain grade and pain-related disability, were inversely correlated with cognitive outcomes, in particular those related to memory. On the other hand, there was a positive relationship between pain chronicity and measures of IQ and verbal memory. The findings of the study are considered below under a number of headings: patient characteristic measures, cognitive outcomes of Phases I and II, and potential limitations associated with the sample and design.

7.4.1 Participant characteristics

A simple comparison of patient and control groups revealed that the proportion of smokers was greater in chronic pain patients than in matched healthy controls. This is in agreement with previous findings of increased rates of cigarette smoking in chronic pain populations (Ekholm et al., 2009; Zvolensky et al., 2009). It is possible that due to the analgesic properties of nicotine (Jones and Dunlop, 2007; Benowitz, 2008), smoking cigarettes could serve as a coping strategy to reduce pain; however, smokers with chronic pain have actually reported higher pain intensities, increased disability and poorer treatment outcomes (Patterson et al., 2012). It is also possible that the increased levels of cigarette smoking are related
to emotional disturbances associated with chronic pain, though experimental findings in this area have been inconsistent (Jamison et al., 1991; Fishbain et al., 2007; Hooten et al., 2011; Patterson et al., 2012). Further investigation in the present sample revealed that smoking status was not correlated with pain intensity measures but did significantly correlate with the depression severity score measured by the PHQ ($r_{pb} = -0.27, p = 0.02$).

The proportion of “non-drinkers” (participants who had not consumed alcohol in over 6 months) was greater in the patient group than in the control group (controls $n = 2$, patients $n = 8$). The time since patients had last consumed alcohol was also significantly greater than that for controls: that is, the average of the duration classifications (1–7 defined above) was higher (indicating a longer duration) in the patients than in the controls. This is broadly in line with the epidemiological study by Ekholm et al. (2009), which found that chronic pain patients were less likely to drink alcohol, but is in contrast with studies that suggest alcohol is used in a “self-medication” capacity in chronic pain (Riley and King, 2009).

The time since last consumption of caffeine was positively correlated with measures of attention, suggesting improved performance with increased duration since participants had last consumed caffeine. This result was unexpected and appears to contradict the established effect of caffeine as a CNS stimulant. However, it is important to note that previous research estimates the elimination half-life of caffeine as 2.5–5 hours (Arnaud, 1987; Culm-Merdek et al., 2005), and the mean duration since last caffeine consumption in the present experiment was between 5 and 6 hours in both patient and control groups. The sample therefore includes both participants in whom caffeine may not yet have reached its peak concentration and participants in whom the effects of caffeine may have been negligible. Furthermore, the duration since last caffeine consumption was a number of days in some participants, and as such, the potential negative effects of caffeine withdrawal on attention may have diminished with increasing time. In addition,
tolerance to the effects of caffeine may develop in heavy users (Fredholm et al., 1999). Another possibility is that participants may have under- or over-estimated the duration since their last caffeine consumption, as the measure was based on self-report. Moreover, in the case of patient participants, interactions between caffeine and other medications could potentially have had a confounding effect on attention.

Depression symptom scores and anxiety scores were significantly elevated in pain patients compared with controls. This result was expected due to the well-documented relationship between chronic pain and affective disorders such as anxiety and depression (Von Korff and Simon, 1996; Gureje et al., 1998), and the previous observations of increased prevalence of these disorders associated with pain (McCracken et al., 1996; Oosterman et al., 2009; Lee et al., 2010; Raftery et al., 2011). Notably, however, despite the exclusion criterion of a diagnosis of major psychiatric illness (including major depression), 50% of participants in the patient group met the PHQ diagnostic requirements for depression (compared with 0% of control participants), with 37% of the total patient group meeting the conditions for a major depressive disorder. These findings suggests a serious under-diagnosis of depressive disorders within this sample. The reasons for the apparent under-diagnosis are unclear. It is likely that these patients initially sought medical attention for pain, with depressive symptoms as a secondary concern. Furthermore, if pain was considered to be the cause of the depressive symptoms, the physician may have been reluctant to clinically diagnose and treat the patient for depression, focusing instead on effective pain management. It is also important to note that the “diagnostic criteria” associated with the PHQ cannot be considered definitive, and diagnosis should always be verified in a clinical setting. Furthermore, it is not clear whether this finding may be generalised to chronic pain patients on a wider scale. In the case of state anxiety, over 70% of patients scored higher than the standard normative scores for working individuals, compared with just 18% in the control group. While state anxiety score is not necessarily indicative of an
anxiety disorder, these results suggest that, within this sample, chronic pain patients were more susceptible to situational anxiety associated with the assessment (or perhaps with their clinic visit) than were controls.

7.4.2 Cognitive measures

7.4.2.1 Phase I

The main hypotheses that patients would perform poorly compared with controls and that the interaction of pain and age would affect all of the cognitive outcome measures tested were both rejected. However, hierarchical multiple regressions revealed an effect of participant group in a number of cognitive outcomes.

A significant effect of participant group (pain patient vs. control) was found in general intelligence as measured by the WAIS dyad, with patients (regardless of their age) having lower IQs than controls. 55% of patients registered IQ scores “below average” (i.e., score < 89), whereas only 16% met this classification in the control group. In the patient group, 42% had IQ scores in the average range and only 3% were in the “above average” (score ≥ 110) range, whereas in the control group, 50% of participants scored in the average range and 34% were considered above average. This is in spite of having matched the patient and control groups on the basis of years of education (which proved difficult, possibly due to the recruitment methods used or because of differences in socioeconomic status) and statistically accounting for years of education in the regression model. A number of previous studies (see Chapter 1) matched patients and controls on the basis of their IQ or statistically controlled for variations in IQ. However, the finding of reduced IQ in chronic pain patients may in fact be evidence of cognitive impairment, possibly related to the neural changes associated with chronic pain as discussed in detail in previous chapters. The WAIS subtests used to estimate IQ were Digit-Symbol Coding and Information, which draw on cognitive resources such as attention and memory (Groth-Marnat, 2009).

The fact that no association was found between pain and performance on the
spatial span forward task is somewhat surprising, given evidence from previous clinical (Dick and Rashiq, 2007; Luerding et al., 2008) and preclinical (see Table 1.3) studies that have shown a negative association between pain and spatial memory. A trend for an effect of group was observed in the reversal of spatial memory assessed in the spatial span task, with patients having lower scores than controls. The spatial span reverse is interesting as it shows some conceptual similarities to the reversal of spatial memory required in the preclinical MWM reversal task. Neuropathic pain has been shown to affect MWM reversal learning here (Chapter 5) and elsewhere (Leite-Almeida et al., 2009). In the spatial span reversal task, clinical participants were required to learn the sequence of blocks, mentally reverse the sequence and plan the response. However, this test is carried out over a much shorter time than the water maze task and does not involve the same relearning component.

A significant effect of the interaction between group and age was observed in the assessment of verbal recognition memory, with the data suggesting a pain-related effect specifically in older participants. Deficits in recognition memory have been shown previously in chronic pain (FM) patients, irrespective of age (Park et al., 2001). Deficits in recognition memory have also been shown in rodent models of chronic pain, both in the study described in Chapter 5 (which was carried out in mid-aged rats) and by other researchers (see Table 1.3). Thus, pain and its interaction with age contributed to a decreased IQ and deficits on specific subtests of memory. Differences between the current results and previous findings may be explained by the specific type of pain investigated here or they may arise from methodological differences.

With regard to attention, an interaction effect was observed in the model for the number of false alarms in the CPT, with older patients having the greatest number of false alarm responses, and a similar effect was evident in the number of random responses, though this was just below the level of statistical significance. A negative relationship between the number of hits and the participant group was
observed. These data suggests that the number of hits was greater in the patient group than in the control group, indicating better task performance by patients. Also in the CPT, there was a significant interaction of age and group in the reaction time to hit responses. Younger controls had shorter reaction times than younger patients but older patients were quicker to respond than older controls. Thus, the CPT results are inconsistent and suggest some pain-related deficits in attention associated with age (increases in false alarms and random responses), but a pain-related increase in the number of correct responses (hits), as well as pain- and age-dependent shortening of reaction time. These results are contrary to previous findings of impaired attention in chronic pain patients (see Table 1.1) and pain-related attentional deficits in rodent models of chronic pain (see Table 1.3). However, it is interesting to note that the results of the present study suggest a general pattern of increased responding in older patients in particular, regardless of whether the responses were correct or incorrect. This may indicate a degree of impulsivity in the patients compared with the control group, which may relate to deficits observed previously on gambling (emotional decision making) tasks. Impulsivity would likely be associated with underlying dysfunction of the PFC, and it is known that there are morphological, neuroplastic and neurochemical changes in the PFC related to chronic pain (see Chapter 1, Section 1.2). Inhibiting responses to false alarms on the CPT involves actively suppressing a learned behaviour, and may be considered a type of reversal learning. Numerous studies have linked the dopaminergic system, particularly in the PFC, with impulsivity measured in reversal-learning tasks. Dopamine depletion in the PFC is associated with impairments in attentional set-shifting in non-human primates (Crofts et al., 2001; Robbins and Roberts, 2007). Pharmacological manipulation of the dopaminergic system also affects reversal learning in both animals and humans, though the results with different agonists and antagonists have been inconsistent (Izquierdo and Jentsch, 2012; Robbins and Arnsten, 2009). Furthermore, disorders associated with impaired impulse control, such as obsessive com-
pulsive disorder, substance-abuse disorders, pathological gambling and attention-deficit/hyperactivity disorder (ADHD), have all been shown to be associated with genetic or functional abnormalities of the prefrontal dopamine system (Fontenelle et al., 2011; Izquierdo and Jentsch, 2012; Robbins and Arnsten, 2009). In addition, Parkinson’s disease, a neurodegenerative disorder characterised by progressive loss of dopaminergic neurons, is also associated with impaired performance on tasks such as the Iowa gambling task and decreases in grey-matter volume in the PFC (Delazer et al., 2009; Ibarretxe-Bilbao et al., 2009). These studies with respect to dopamine may suggest a link between the present CPT findings, previous reports of altered dopaminergic transmission in chronic pain (Wood and Holman, 2009; Wood et al., 2007a,b), and the finding of increased levels of dopamine and dopamine metabolites in the cerebral cortex and thalamus presented in Chapter 6.

The absence of any effects of pain, or of the pain-age interaction, on WCST outcome measures was unexpected. There is a strong body of literature to suggest that executive-type functions in pain patients and analogous cognitive flexibility in rodents are impaired in the presence of chronic pain (see Chapter 1 and Chapter 5). There are, however, a number of studies in which pain did not show an effect on executive function, specifically in the WCST (Suhr, 2003) and in a battery of tests that included digit-span backward, category fluency, Knox’s Cube Imitation (similar to spatial span) and incomplete figures (Scherder et al., 2008). As mentioned in Section 7.1 above, Oosterman et al. (2009) showed an improvement in executive function (measured using a spatial working memory task, the Trail Making Task B, and the colour/word Stroop task) related to increased self-reported pain specifically in older chronic pain patients. If this were the case in the present study, an effect of the interaction between group and age on WCST outcome measures would be anticipated, but this was not observed. Performance in general (in both patients and controls) on the WCST was relatively poor, with standard score average for the total sample in the range 89–93, compared with the standard score average of 100. Thus, the present sample may not be a good
representation of the standard sample and, as such, the demographic corrections applied may not have been strictly valid.

7.4.2.2 Phase II

The number of painful areas reported appeared to be a good predictor of verbal memory, with scores decreasing as the pain diffuseness increased. Pain chronicity was found to be a positive predictor of IQ and verbal memory. This result is somewhat surprising, even though previous assessments of the extent to which pain chronicity, or duration of chronic pain illness affect cognitive outcomes have produced contrasting results. Eccleston (1994) and Alanoglu et al. (2005) found that pain chronicity was not associated with differences in cognitive function, whereas other studies have shown that duration of illness (migraine, diabetes and FM, CRPS and chronic back pain) was inversely related to cognitive performance (Apkarian et al., 2004a; Calandre et al., 2002; Ryan, 2005; Verdejo-García et al., 2009). A positive correlation as shown in this study has not, therefore, been demonstrated previously, but it may represent a habituation or compensation mechanism induced in states of prolonged chronic pain. Further investigation of this theory is warranted. Pain intensity was not generally associated with alterations in cognitive function, which suggests that other aspects of the pain experience may contribute to changes in cognition. Chronic pain grade and pain-related disability negatively affected aspects of attention, suggesting that this cognitive domain may be more susceptible to the affective or disabling dimensions of pain. Interactions between pain variables and age also made contributions to memory and attention outcomes, again indicative of an important role for age in the interaction of pain and cognition. Therefore, although the second set of hypotheses was also rejected, but there are a number of exceptions related to specific cognitive outcomes.
7.4.3 Limitations

In discussing the findings of the present study it is important to acknowledge a number of limitations. A sample size of 76 could be considered relatively small for observational research; however, sample sizes of this order have been used previously in pain/cognition research (see Table 1.1). The pain variable measures and the records of current medication use relate only to the patient subset of participants and so sample size is further reduced for these measures ($n = 38$). Therefore, effects of medication on cognitive outcomes cannot be ruled out completely, notwithstanding the application of appropriate statistical control measures.

The apparent under-diagnosis of depression within the present sample of chronic pain patients means that the effects of depression could be controlled for statistically, but not experimentally. Depression was strongly correlated with group ($r_{pb} = -0.838$, $p < 0.01$), indicating a higher depressive symptom score in the patients, and was also significantly correlated with a number of cognitive outcomes (see Table 7.4). In spite of this, significant contributions of group and age-group interaction to some cognitive scores were found, over and above the contribution of depression. It is unlikely that the extent to which the sample represents the chronic pain population as a whole is compromised by the presence of undiagnosed depression, since it is known that an increased prevalence of depression associated with chronic pain. Materials for cognitive testing were selected based on their potential to contribute to the translational model of pain-related cognitive effects. It is not known whether this series of tasks has been used in combination previously, and it is possible that the order in which the tests were administered may have affected task performance. This is one possible explanation for the relatively poor WCST outcomes (see previous section), as this task was one of the last to be administered. Nevertheless, no decrease in effort or motivation was noted in observation of participants during the assessment period.
7.4.4 Conclusions

This study provides partial support for the theory that pain affects cognition and that the relationship is influenced by age. The cognitive outcomes affected were mainly within the domains of memory and attention, with IQ and psychomotor speed also affected. The results show some parallels with findings reported in the literature, both clinical and preclinical. However, some of the findings were in direct contrast to those reported previously. Further research is required to determine whether this set of outcomes represents a signature of cognitive performance in neuropathic and radicular pain, or whether the divergent results may be explained by differences in the methodologies.
Chapter 8   General Discussion

The prevalence of chronic pain and its impact both on the individual and on the wider community make it a significant healthcare problem. Chronic pain is not only associated with disabling limitations on physical function but can also have marked psychological consequences. Despite some evidence that pain negatively affects cognition, the relationship between the two is poorly understood. Impaired cognition in chronic pain patients would likely reduce their quality of life, impede rehabilitation and increase suffering. Understanding the interaction between pain and cognition is therefore of considerable therapeutic significance, as well as being of interest both physiologically and psychologically. The studies presented in this dissertation aimed to advance understanding of the effect of chronic pain on cognition through a series of empirical preclinical and clinical investigations. To that end, Chapters 3–6 addressed the need for an animal model demonstrating cognitive impairment associated with pain to investigate the neural mechanisms underlying the relationship between chronic pain and cognition, while Chapter 7 assessed cognitive function in a clinical sample of chronic neuropathic or radicular pain patients.

The work presented herein describes the first characterisation of cognitive function in rats that had undergone L5–L6 spinal nerve ligation (SNL) surgery as a model of chronic neuropathic pain (Kim and Chung, 1992) in either early adulthood or mid-age, using a range of classical behavioural tests. A noteworthy advance was made with the establishment and validation of a novel passive-avoidance paradigm for the assessment of aversive learning and memory in the SNL and other models of chronic pain. The studies provide some evidence for age- and domain-specific deficits in cognitive performance associated with the SNL model.
of neuropathic pain. Furthermore, the studies identify pain-related alterations in synaptic plasticity in cognitive-associated brain regions in the SNL model, including the hippocampus, the prefrontal cortex and the amygdaloid cortex, which may contribute to the observed impairments in cognitive behaviour. Pain-related alterations in monoaminergic transmission have also been identified in the SNL model. The clinical study expands on previous investigations that have shown cognitive impairment in chronic pain patients, with neuropathic or radicular pain patients found to perform poorly compared with controls on measures of intelligence and memory but not on measures of attention or executive function. Moreover, significant interactions between these cognitive outcomes and age were also observed, highlighting the influence of age on the relationship between chronic pain and cognition.

The first results chapter (Chapter 3) describes an initial assessment of cognitive function in young adult rats that had undergone SNL surgery as a model of neuropathic pain. These rats expressed characteristic neuropathic pain-like behaviours (mechanical allodynia and thermal hyperalgesia) in the two-week period post surgery, in line with previous studies (Chaplan et al., 1994; Kim and Chung, 1992). SNL surgery also resulted in a decrease in IB4 staining in the ipsilateral spinal cord at the level of the L5 and L6 spinal nerves, a hallmark of peripheral nerve injury (Munglani et al., 1995). Cognitive function was assessed using a series of behavioural tests: the novel object recognition paradigm, the T-maze spontaneous alternation task and the acquisition training and probe tasks in the Morris water maze. In this experiment, SNL surgery was not associated with any significant behavioural deficits in recognition memory or spatial learning and memory assessed in these paradigms. This finding was in contrast with previous reports of pain-related cognitive impairment in the literature (Boyette-Davis et al., 2008; Cain et al., 1997; Hu et al., 2010; Ji et al., 2010; Kodama et al., 2011; Leite-Almeida et al., 2009; Lindner et al., 1999; Millecamps et al., 2004; Pais-Vieira et al., 2009a,b; Ren et al., 2011). There were, however, a number of important
experimental differences including the type of pain model under investigation, the age of the experimental subjects, the time points selected for cognitive testing and the specific cognitive tests used. Cognitive function had not previously been investigated in the SNL model. Therefore, the results of this experiment may be interpreted as evidence for a lack of cognitive impairment in the SNL model. However, based on the available literature regarding cognitive function in other neuropathic pain models, it was decided that further investigation was warranted.

The primary objective of the studies carried out in Chapter 4 was to assess whether aversive learning and memory were affected in rat models of chronic pain. The established methods for investigating aversive learning and memory were considered to be unsuitable for use in models of chronic pain affecting the rats’ hind-paws, as they predominantly involve noxious foot-shock as a context-conditioning aversive stimulus (Elrod and Buccafusco, 1988; Graham and Buccafusco, 2001; Holloway and Wansley, 1973), the response to which may be confounded in chronic pain models. Thus, alternative aversive stimuli were investigated for their ability to induce a passive-avoidance response in a light/dark box, which is indicative of intact aversive memory. Specifically, exposure to an ultrasonic tone and aversive air-puff were investigated. Ultrasound (21 kHz square wave at a volume of 100 ±10 dB for one minute) was not associated with a significant passive-avoidance response. This result was contrary to that of Dokla et al. (1989), who demonstrated a passive-avoidance response using ultrasound at a similar frequency and intensity. However, strain differences in the response to ultrasound, as described previously (Commissaris et al., 2000), may have accounted for this difference. A brief exposure to air-puff, on the other hand, significantly increased the step-through latency to the dark compartment of the light/dark box in the retention trial, indicating a passive-avoidance response. This response could be reversed by pre-acquisition treatment with a known inhibitor of cognitive function, the muscarinic cholinergic antagonist scopolamine. Thus, this novel paradigm was valid as a test of aversive learning and memory which did not require use of noxious foot-shock. Aversive
learning and memory in the air-puff-induced passive-avoidance paradigm were not affected in the SNL model of neuropathic pain or in an alternative model, the CFA model of chronic inflammatory pain. The rationale for investigating aversive memory in models of chronic pain was based on the involvement of common neuroanatomical regions in pain and aversion (Ambrogi Lorenzini et al., 1997; Apkarian et al., 2005; LeDoux, 1993; Lorenzini et al., 1996), and on the fact that other behavioural phenomena, such as fear- and stress-induced analgesia (Butler and Finn, 2009; Ford et al., 2008), occur as a result of interaction between neural pain and aversion processing systems. Based on our results, aversive learning and memory do not appear to be affected in models of chronic pain, although the result may again have been influenced by experimental factors (for example, the age of the rats).

Cognitive impairment in a different model of chronic neuropathic pain was found to be age-specific, occurring only in mid-aged rats (Leite-Almeida et al., 2009). Therefore, in Chapter 5, aversive learning and memory were also assessed in rats of a similar age to the mid-aged cohort in the Leite-Almeida et al. (2009) study, but again the results suggested that there was no effect of SNL surgery on the passive-avoidance response. Previous work by Suzuki et al. (2007) showed that foot-shock-induced passive avoidance was not impaired in a mouse model of neuropathic pain, and rats’ ability to acquire an active-avoidance response in place escape/avoidance paradigms was also unaffected in models of chronic pain (LaBuda and Fuchs, 2000; Pedersen and Blackburn-Munro, 2006), findings which are in agreement with the results presented here. Thus, aversive memory appears to be minimally affected by chronic pain under a variety of experimental conditions. In spite of this, the establishment and validation of the air-puff passive avoidance paradigm is important as it may be ethically preferable to foot-shock-induced passive avoidance paradigms and may be more suitable for use in assessment of cognitive function in situations where pain sensitivity is altered (for example, in disease models or following administration of analgesic compounds).
Based on the recent finding that the SNI model of neuropathic pain was associated with cognitive deficits, but specifically in mid-aged rats (aged approximately 9 months; Leite-Almeida et al., 2009), a further characterisation of cognitive performance in the SNL model in mid-aged rats was carried out. Recognition memory and spatial learning and memory were again examined using tasks similar to those used in Chapter 3. In addition, cognitive flexibility was tested using a reversal task in the Morris water maze. The type of functioning assessed by this task is thought to be analogous to executive function in humans. Impairment of executive function in chronic pain patients has been demonstrated previously in attentional interference and switching tasks (Bosma and Kessels, 2002; Eccleston, 1994; Grisart and Plaghki, 1999; Karp et al., 2006; Ryan et al., 1993; Weiner et al., 2006), in the Wisconsin Card Sorting Test (Verdejo-García et al., 2009), and in process dissociation tasks (Grisart and Van der Linden, 2001). Furthermore, this type of cognition is heavily dependent on the prefrontal cortex (Kouneiher et al., 2009; Robbins, 1996), a region also involved in the experience of pain (Apkarian et al., 2005). Therefore, performance in this task in the SNL model was of particular interest.

In this experiment, the SNL model was not only associated with characteristic allodynia and hyperalgesia, but also with cognitive impairment. There was significantly reduced discrimination of the novel object in the novel object recognition test, suggesting impaired recognition memory, and reversal learning and adaptive memory were impaired relative to sham controls in the Morris water maze reversal task, indicative of a deficit in cognitive flexibility. The deficit in reversal suggests that, similar to the clinical situation, the interference effect of pain may be dependent on the complexity of the cognitive task. Therefore, future work in the context of chronic pain models could investigate performance in demanding cognitive tasks such as the five-choice serial reaction time test (5CSRTT) (Robinson, 2012), attentional set-shifting (Birrell and Brown, 2000), and touchscreen discrimination tests (Bussey et al., 2008), tasks in which the PFC is heavily involved.
The behavioural results presented in Chapter 5 are remarkably similar to those of Leite-Almeida et al. (2009), who showed that SNI (a neuropathic pain model involving transection of the common peroneal and tibial nerves) was associated with impaired cognitive flexibility, also in mid-aged rats. Thus, for the first time, behavioural deficits associated with peripheral nerve injury had been demonstrated across different pain models, providing convincing evidence that the impairments were pain-related, possibly dependent on the type of pain, and influenced by age. However, it is also important to note that not all preclinical animal studies demonstrating pain-related impairments of cognition were associated with the same age component (Cain et al., 1997; Hu et al., 2010; Kodama et al., 2011; Lindner et al., 1999; Ren et al., 2011). In the clinic, the effect of pain on cognition has been studied predominantly in mid-aged adults, as this is the age group that most commonly seeks primary care. The impact of age on the relationship between chronic pain and cognitive function has, therefore, been under-researched. A number of studies have shown that chronic pain had a negative effect on cognition specifically in older subjects (Buckalew et al., 2010, 2008; Karp et al., 2006; Oosterman et al., 2009; Scherder et al., 2008). Only one study has investigated directly the effect of ageing on the pain/cognition relationship in humans (Oosterman et al., 2011), but no interaction between age and pain-related memory impairment was shown. The behavioural results presented in Chapter 5 suggested the establishment of a putative model of pain-related cognitive impairment and, as such, potential underlying mechanisms were further investigated. As described in Chapter 1, synaptic plasticity represents a link between chronic pain and cognition, and changes in plasticity may account for the cognitive disturbances seen in chronic pain patients and in the present model of chronic neuropathic pain. Synaptophysin was measured as a marker of the density of synaptic connections and was found to be increased in the prelimbic region of the mPFC and the CA1 region of the hippocampus in SNL rats compared to sham controls. These regions are involved in spatial working memory, cognitive flexibility and spatial novelty, and a variety
of structural and functional changes in these areas have been shown in models of chronic pain in both humans and animals (Apkarian et al., 2004b; Ji et al., 2010; Kodama et al., 2007; Luerding et al., 2008; Metz et al., 2009; Mutso et al., 2012; Seminowicz et al., 2009; Zhao et al., 2009; Zimmerman et al., 2009). Alterations in the expression of synaptophysin in these regions were therefore expected in the present study, although given the behavioural results showing cognitive deficits, a decrease in synaptic connectivity might have been predicted. However, the cellular composition of both regions is complex, and it is possible that an increase in synaptophysin could be associated with a net inhibitory effect and could therefore correlate with the behavioural results observed. The results are, nonetheless, in direct contrast to the findings of Ren et al. (2011), who showed a decrease in the number of synaptophysin-positive terminals in the CA1 region of SNI rats. It is important to note that, while SNI and SNL both model peripheral neuropathic pain, the site and type of injury are different. Furthermore, the behavioural tests used to assess cognition, the time of sacrifice post surgery, and the age of the rats differed between the present experiment and that of Ren et al. (2011). While it is possible that SNI and SNL have contrasting effects on synaptophysin expression in the CA1, it is puzzling that both would result in the same behavioural effects. It is clear that further investigation of the type of neurons labelled by synaptophysin is warranted.

In Chapter 6, we aimed to replicate the behavioural findings of impaired cognition in the SNL model in mid-aged rats and to investigate whether such impairments could be reversed by alleviating the neuropathic pain symptoms with amitriptyline. Amitriptyline significantly attenuated SNL-induced thermal hyperalgesia, but not mechanical or cold allodynia, similar to previous studies (Esser et al., 2001; Esser and Sawynok, 2000; Pradhan et al., 2010). Deficits in the novel object recognition paradigm and in the Morris water maze reversal task, as had been demonstrated in Chapter 5, were not reproduced in this experiment. The reason(s) for this discrepancy is unclear as the experimental design was broadly
similar to that used in Chapter 5. Amitriptyline was administered in drinking water so as to avoid confounding effects of additional handling or stress associated with alternative routes of administration. The main difference in the study design related to the sequence and frequency of behavioural testing and further investigation of the effect of such factors on cognitive behavioural outcomes is therefore warranted. The result calls into question the robustness and reproducibility of the putative model described in Chapter 5 and as such further experiments should be carried out to confirm its validity. Despite the fact that behavioural deficits in cognition were not observed, post-mortem analysis allowed for the investigation of a number of other important research questions. These related to the effect of neuropathic pain induced by SNL surgery levels on monoamines and expression of synaptophysin in the brain and spinal cord, and to the mechanism of action of amitriptyline as an antihyperalgesic. The SNL model in mid-age rats was found to be associated with alterations, primarily in dopaminergic transmission, in supraspinal brain regions including the thalamus, and the amygdaloid and cerebral cortices. Abnormal dopaminergic transmission has been shown previously, both in chronic pain patients and in models of chronic or persistent pain (Finn et al., 2006; Ford et al., 2008; Pais-Vieira et al., 2009b; Wood et al., 2009, 2007b). In the present study, pain-related changes in monoamine levels were, in some cases, attenuated by treatment with amitriptyline, suggesting that changes in brain levels of monoamine may contribute to the expression and maintenance of neuropathic pain-like behaviours in the SNL model. Serotonin tended to be decreased in the dorsal spinal cord of SNL rats compared with sham controls. This finding contradicts that of Nakajima et al. (2012), who found that serotonin and noradrenaline were increased in the spinal cord of nerve-injured animals, though this group used the SNI model and potential differences between the SNI and SNL models should again be noted. The only alteration observed in synaptophysin expression related to the SNL model was a lateralised increase in the amygdaloid cortex. This finding differs from the results presented in Chapter 5. In that chap-
ter, synaptophysin was found to be increased in SNL in both the hippocampus and the PFC, but no such alterations were observed in the Chapter 6 study. Differences in the measurement technique may account for the contrasting results. While some regional effects of amitriptyline *per se* on synaptophysin expression and monoamine levels were observed, a greater effect of amitriptyline treatment on these transmitters might have been expected in this experiment, as the antidepressant action of amitriptyline is partly attributed to its action as an inhibitor of the reuptake of serotonin and noradrenaline. A typical antidepressant dose of amitriptyline in a rat model of depression was 10 mg/kg administered subcutaneously (Kelly et al., 1997; Song and Leonard, 2005). In the present experiment, a similar dose was administered in the drinking water, but due to the low oral bioavailability of amitriptyline (Bae et al., 2009), the dose reaching systemic circulation would have been much lower. The antihyperalgesic mechanism of action of amitriptyline may therefore be different to its antidepressant mechanism, and may be mediated by peripheral targets rather than acting at central monoaminergic synapses.

The aims of the clinical study described in Chapter 7 were to expand on the existing clinical literature describing the effect of chronic pain on cognition and to determine the translational validity of the present preclinical findings. Cognitive function was assessed across four broad cognitive domains (verbal memory, spatial memory, attention and executive function) in patients with chronic neuropathic or radicular pain and in age- and gender-matched control participants. Estimated IQ scores were found to be lower in chronic pain patients than in controls, even after statistically accounting for the number of years of education. Since the tests used to estimate IQ require the use of cognitive resources such as memory and attention (Groth-Marnat, 2009), this finding may represent a crude expression of pain-related impairment of cognitive function. A strong trend for a deficit in performance on the spatial span-reverse task was observed in the patient group relative to controls. This task shows some conceptual similarity with the Morris
water maze spatial reversal task in rodents, and this trend therefore mirrors the preclinical behavioural finding of impaired spatial reversal in the SNL neuropathic pain model reported in Chapter 5. Verbal recognition memory was predicted by the interaction of pain and age, with the data suggesting a negative effect of pain on this measure specifically in older patients. This result also shows some similarities with the preclinical studies presented here, in which SNL surgery was associated with impaired recognition memory in mid-aged rats but not in young adult rats (Chapters 3 and 5). The interaction between pain and age was also significant for measures of attention. Responding in the attention task was increased in the patient group but this increase was irrespective of whether the responses were correct or incorrect (both false-alarm and random responses), particularly when age was considered as a factor. Thus, a deficit in attention was not detected in this study but the pattern of responding may suggest an impulsive tendency in the patient group. Chronic pain patients have previously shown impaired emotional decision making on tasks such as the Iowa Gambling Task (IGT) (Apkarian et al., 2004a; Verdejo-García et al., 2009). Performance on the IGT is associated with facets of impulsive behaviour, including a lack of premeditation and novelty-seeking (Zermatten et al., 2005). Furthermore, in rodent gambling tasks analogous to the IGT, deficits have also been shown in models of chronic pain (Ji et al., 2010; Pais-Vieira et al., 2009b). Impulsive behaviour involves neural GABAergic, glutamatergic and monoaminergic mechanisms in rodents (Murphy et al., 2012; Robinson, 2012; Bari et al., 2009). Interestingly, the present studies have demonstrated increased synaptophysin expression (possibly indicating altered synaptic plasticity) and regional alterations in monoamines in mid-aged SNL rats, although the implications of this finding for impulsive behaviour were not investigated. As discussed in Section 7.2.4.1, dopaminergic signalling is critically involved in tasks measuring inhibitory control and reversal learning. This neurotransmitter system may, therefore, be of interest in future studies of pain, impulsivity and general cognitive function. Impulsive behaviour in rodent models of chronic pain could
be further investigated using behavioural tasks such as 5CSRTT (Robinson et al., 2009) and stop-signal paradigms (Bari et al., 2009), in addition to the gambling tasks commonly used. These and other emotional decision-making tasks are particularly important because they translate well to the clinical situation (Anderson et al., 2012; Zeeb et al., 2009).

Surprisingly, an effect of chronic pain was not observed on the WCST test of executive function. Chronic pain patients have performed poorly compared with controls previously on this (Verdejo-García et al., 2009) and other tests of executive function (Bosma and Kessels, 2002; Eccleston, 1994; Grisart and Plaghki, 1999; Karp et al., 2006; Ryan et al., 1993; Weiner et al., 2006; Grisart and Van der Linden, 2001). Preclinical evidence here and elsewhere (Leite-Almeida et al., 2009) also suggests impairment of executive-type cognitive functioning in models of chronic pain. However, there are also cases in the literature where chronic pain was not associated with impairment of executive functions (Scherder et al., 2008; Suhr, 2003) or was in fact associated with improved executive function (Oosterman et al., 2011). Thus, it would appear that both clinically and preclinically, further investigation of executive functioning in chronic pain is necessary.

The main contribution of the work described in this dissertation is the advancement of understanding of the complex relationship between pain and cognition, the modulation of this relationship by age, and the potential underlying neural mechanisms. The development of the novel air-puff induced passive-avoidance paradigm is also noteworthy and may be relevant to this and other fields of behavioural neuroscience research. The work does raise significant questions that will require continued research in the future. Specifically, the reproducibility of cognitive deficits in the SNL model in mid-aged rats should be further scrutinised. It is also important to establish the cell types involved in altered synaptic plasticity in this model. The clinical study provided some insights into the effect of neuropathic or radicular pain on cognitive behaviour across a number of cognitive
domains. However, the study is limited by the relatively small sample size and a study of similar design but on a larger scale would likely provide much more definitive conclusions. The inclusion of an imaging component in such a study would also allow for concurrent investigation of the neural mechanisms involved.

The results presented in this dissertation are preliminary, in that further research is required to integrate the findings into a working model of pain-related cognitive impairment. The studies herein do, however, identify a number of promising areas for future investigation. The behavioural findings of impaired novel object recognition and reversal learning in the SNL model of neuropathic pain in mid-aged rats strongly suggests a dysfunction of the PFC in this model. This is supported by the finding of altered synaptophysin expression in the mPFC in cognitively impaired SNL rats. The PFC is reciprocally connected with a large number of cortical and limbic brain regions and appears to be pivotal in the interaction of pain and cognition. The SNL model was also associated with alterations in monoamines in discrete brain regions, with the dopaminergic system showing the greatest modulation. The levels of dopamine and its metabolite DOPAC were found to be elevated in the cerebral cortex and thalamus of SNL rats. A delicate balance of dopaminergic activity in regions such as the PFC is required for normal cognitive functioning and may follow an inverted-U-shaped dose response (Robbins and Arnsten, 2009). Altered dopamine receptor binding has been shown in chronic pain syndromes such as fibromyalgia, with patients having decreased tonic dopaminergic activity (in the brainstem, thalamus and limbic cortex), and showing altered dopaminergic responsivity to noxious stimuli (in the basal ganglia) (Wood et al., 2007a,b). Concurrently, alterations in dopamine signalling are implicated in a wide variety of neurological disorders of which cognitive impairments are characteristic, including schizophrenia, ADHD, Alzheimer’s disease, Parkinson’s disease, OCD and substance-abuse disorders. Dopaminergic alterations include changes in D1 and D2 receptor binding (measured by PET) and genetic polymorphisms, for example in the dopamine metabolic enzyme COMT.
(Robbins and Arnsten, 2009), and these alterations result in functional deficits including impaired working memory, emotional decision making, reversal learning and inhibitory control (Izquierdo and Jentsch, 2012; Robbins and Arnsten, 2009). Thus, the cognitive deficits associated with chronic pain may be related to altered dopamine function in discrete brain regions. Moreover, cognitive impairment in chronic pain may be related to other cognitive disorders through a common mechanism of dopaminergic dysfunction. Dopamine is also thought to be important in neuronal integrity (Bozzi et al., 2000; Bozzi and Borrelli, 2002), and in synaptic plasticity by enhancing synaptogenesis and neural sprouting (Stroe-mer et al., 1998). The increase in tissue levels of dopamine may therefore be connected with the increased expression of synaptophysin observed in the mPFC and hippocampus of SNL rats seen in Chapter 5. Further research is required to confirm this hypothesis and to determine how this relates to the behavioural outcomes observed.

The high incidence of undiagnosed depressive symptoms in our clinical sample suggests that it may be inappropriate, or even detrimental, to control for such symptoms in studies investigating the effect of chronic pain on cognitive function. Chronic pain is associated with a complex multidimensional symptom profile and appears to be intrinsically linked with affective disorders. This theory is supported by the fact that depressive-like symptoms are also observed in rodent models of chronic pain (Gonçalves et al., 2008; Leite-Almeida et al., 2009; Suzuki et al., 2007). There is evidence that anxiety or depressed mood can be initiated or exacerbated by pain, and that conversely affective disorders may precipitate chronic pain (Bair et al., 2003; Dworkin and Gitlin, 1991; Gureje et al., 2001). Impairments of cognitive function are common in depressive and anxiety disorders (Austin et al., 2001; Castaneda et al., 2008; Eysenck et al., 2007; Hindmarch, 1998). Reward- or motivation-based tasks are particularly affected in depression (Nestler and Carlezon, 2006). Interestingly, the symptoms of anxiety and depression, and the cognitive impairments associated with these disorders, are also
thought to involve fronto-cortical dopamine signalling, suggesting that disruption of the dopaminergic system may be shared in chronic pain and affective disorders. The interrelationship between the PFC, the dopaminergic system and synaptic plasticity, and the manner in which it contributes to the complex comorbidity of chronic pain and affective disorders seem to be among the most promising areas for further research.

Chronic pain has debilitating physical and psychological consequences, and management of its effects represents a major challenge. The mechanisms underlying the effects of chronic pain are currently poorly understood. The present work has examined the neuropsychological and behavioural impact of chronic pain, specifically in the area of cognitive function. The elucidation of a distinct and reproducible profile of the effects of pain on cognitive function will allow for more effective therapeutic strategies for chronic pain sufferers.
Bibliography


BIBLIOGRAPHY


Appendix A  Buffers and Solutions

A.1 Transcardial perfusion

Phosphate buffer (PB)

Stock A

Sodium dihydrogen phosphate monohydrate (NaH$_2$PO$_4$·H$_2$O, MW = 137.99)
0.2 M = 27.598 g in 1 l distilled H$_2$O

Stock B

Disodium hydrogen phosphate dihydrate (Na$_2$HPO$_4$·2H$_2$O, MW = 177.99)
0.2 M = 35.598 g in 1 l distilled H$_2$O

⇒ For 1000 ml (1 l) PB :
95 ml stock A + 405 ml stock B + 500 ml distilled H$_2$O = 1000 ml PB

Fixative: 4% paraformaldehyde (4 l)

Heat 1.5 l of distilled H$_2$O to 60°C.
Add 160 g of paraformaldehyde powder.
Add a few NaOH pellets in order to dissolve paraformaldehyde.
Stir until clear.
Fill to 2 l with distilled H$_2$O.
Stir until clear.
Add 2 l of 0.2 M PB to give a final volume of 4 l and final PB concentration of 0.1 M.
Cool to 4°C and adjust pH to 7.4.
**APPENDIX A. BUFFERS AND SOLUTIONS**

**Heparinised saline**

5000 units of heparin is added per 1 l saline (0.9% NaCl in distilled H₂O).

Stock heparin contains 5000 units per ml, such that 1 ml of heparin is added per litre of saline.

**0.1 M Phosphate-buffered saline (PBS)**

**Stock A**

Sodium dihydrogen phosphate monohydrate (Na₂HPO₄·H₂O, MW = 137.99)

0.1 M = 13.799 g in 1 l distilled H₂O

**Stock B**

Disodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O, MW=177.99)

0.1 M = 17.799 g in 1 l distilled H₂O

⇒ For 1000 ml (1 l) PB :

95 ml stock A + 405 ml stock B + 500 ml distilled H₂O = 1000 ml PB

0.1 M PB prepared according to the above and 0.9 g of saline added per 100 ml of PB.

Alternatively:

PBS tablets (Sigma-Aldrich, Dublin, Ireland) dissolved in distilled water.

1 PBS tablet per 200 ml water gives 0.1 M PBS pH 7.4.

**25% w/v sucrose solution with 0.1% sodium azide**

For 1 l, 250 g of sucrose and 1 g sodium azide dissolved in distilled H₂O.
A.2 Histology and immunohistochemistry

5% w/v sucrose solution with 0.1% sodium azide

For 1 l, 50 g of sucrose and 1 g sodium azide dissolved in distilled H$_2$O.

0.1 M Phosphate-buffered saline (PBS)

Stock A

Sodium dihydrogen phosphate monohydrate (NaH$_2$PO$_4$.H$_2$O, MW = 137.99)

0.1 M = 13.799 g in 1 l distilled H$_2$O

Stock B

Disodium hydrogen phosphate dihydrate (Na$_2$HPO$_4$.2H$_2$O, MW=177.99)

0.1 M = 17.799 g in 1 l distilled H$_2$O

⇒ For 1000 ml (1 l) PB

95 ml stock A + 405 ml stock B + 500 ml distilled H$_2$O = 1000 ml PB

Alternatively:

PBS tablets (Sigma-Aldrich, Dublin, Ireland) dissolved in distilled water.

1 PBS tablet per 200 ml water gives 0.1 M PBS pH 7.4.

Antibody diluent and blocking solution

PBS 25 ml

0.2% Triton-X 50 µl

3% normal rabbit serum 750 µl

Primary antibody

Mouse anti-synaptophysin diluted 1:1000 in antibody diluent solution.

Secondary antibody

AF488-conjugated rabbit anti-mouse IgG, diluted 1:100 in antibody diluent solution.
A.3 Western immunoblotting

**4X Sample buffer**: stored at −20°C

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>0.92 g</td>
</tr>
<tr>
<td>1 M Tris-HCl pH 6.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>1% bromophenol blue (PBS)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

Add 50 µl 2-mercaptoethanol to aliquot in fume hood when ready to use.

**Lysis buffer**: stored at 4°C

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 mM sodium β-glycerophosphate</td>
<td>24.98 g</td>
</tr>
<tr>
<td>1 mM dithiothreitol</td>
<td>0.155 g</td>
</tr>
<tr>
<td>1 mM sodium fluoride</td>
<td>0.042 g</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Adjust pH to 7.6 at 4°C.

Protease inhibitor (Sigma-Aldrich, Dublin, Ireland, Cat. no. P8340) was added prior to use, 10 µl per 10 ml of lysis buffer.

**0.5 M Tris, pH 6.8**: stored at 4°C

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma-base</td>
<td>12.11 g</td>
</tr>
<tr>
<td>Distilled H₂O (on stirrer)</td>
<td>200 ml (final)</td>
</tr>
</tbody>
</table>

Adjust pH with HCl to 6.8.
1.5 M Tris, pH 8.8: stored at 4°C

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma-base</td>
<td>90.82 g</td>
</tr>
<tr>
<td>Distilled H$_2$O (on stirrer)</td>
<td>500 ml (final volume)</td>
</tr>
</tbody>
</table>

Adjust pH with HCl to 8.8.

20% SDS

Sigma-Aldrich, Dublin, Ireland (Cat. No. 05030)

10% Ammonium persulphate

1 g ammonium persulphate in 10 mls H$_2$O

5X Running buffer: stored at room temperature

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma-base</td>
<td>15 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>108 g</td>
</tr>
<tr>
<td>SDS (20%)</td>
<td>25 ml</td>
</tr>
<tr>
<td>Distilled H$_2$O</td>
<td>1000 ml (final volume)</td>
</tr>
</tbody>
</table>

1X Transfer buffer: stored at 4°C

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>2000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma-base</td>
<td>6.06 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>28.8 g</td>
</tr>
<tr>
<td>Methanol (20%)</td>
<td>400 ml</td>
</tr>
<tr>
<td>Distilled H$_2$O</td>
<td>2000 ml (final volume)</td>
</tr>
</tbody>
</table>
APPENDIX A. BUFFERS AND SOLUTIONS

**10X Tris-buffered saline (TBS):** stored at 4°C

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>1 000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mM Trizma-base</td>
<td>24.23 g</td>
</tr>
<tr>
<td>1.37 M NaCl</td>
<td>80.06 g</td>
</tr>
<tr>
<td>Distilled H₂O (dissolve in on stirrer)</td>
<td>800 ml</td>
</tr>
<tr>
<td>Adjust pH to 7.6</td>
<td></td>
</tr>
<tr>
<td>Distilled H₂O (add after adjusting pH)</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

**Blocking solution:** stored at 4°C

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 g milk formula (Aptamil)</td>
<td>5 g</td>
</tr>
<tr>
<td>Tween 20 (0.05%)</td>
<td>50 µl</td>
</tr>
<tr>
<td>1X Tris-buffered saline (TBS)</td>
<td>100 ml (final volume)</td>
</tr>
</tbody>
</table>

**Washing solution:** stored at 4°C

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 20 (0.05%)</td>
<td>200 µl</td>
</tr>
<tr>
<td>1X TBS</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Primary antibody diluent:** stored at 4°C

Ingredients: Same as Blocking solution.

**Secondary antibody diluent:** stored at 4°C

Ingredients: Same as Blocking solution.
**Stripping buffer**

25 mM glycine-HCl pH 2, 1% SDS

Add 1.876 g glycine to 950 ml water.

Adjust pH to 2.0 with HCl.

Add 50 ml of 20% SDS.

**12% SDS-PAGE resolving gel**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>1 × (total volume 10 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>3.29 ml</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% APS (fresh)</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

**A.4 High-performance liquid chromatography (HPLC)**

**Mobile phase buffer**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>4 l final volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M citric acid monohydrate</td>
<td>80.056 g</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate monohydrate (NaH$_2$PO$_4$.H$_2$O)</td>
<td>55.196 g</td>
</tr>
<tr>
<td>1.4 mM octane sulphonic acid</td>
<td>1.211 g</td>
</tr>
<tr>
<td>0.1 mM EDTA disodium salt</td>
<td>0.149 g</td>
</tr>
</tbody>
</table>

Each ingredient was dissolved in turn in nanopure water (resistivity > 16.7 MΩ.cm). 400 ml of HPLC grade methanol was added and the pH was adjusted to 3.5.
Standards

Stock solutions of 10 mg/10ml (10 mg of pure compound, not stabilised salt) of each monoamine/metabolite were prepared. The amount of each compound required was as follows:

- L (-)-noradrenaline (+)-bitartrate salt monohydrate: 18.75 mg
- L-DOPA: 10.00 mg
- DOPAC: 10.00 mg
- Dopamine hydrochloride: 12.38 mg
- 5 – HIAA: 10.00 mg
- HVA: 10.00 mg
- Serotonin creatinine sulphate complex: 22.00 mg
- N_ω_-methyl-5-hydroxytripamine oxalate salt: 14.70 mg

Each dissolved in 10 ml of mobile phase buffer.

200 ng/20 µl standards

100 µl of each of the above standards diluted 1:100 to a volume of 10 ml in mobile phase buffer.

Standard Mix

100 µl of all 200 ng/20 µl standards made up to 10 ml with mobile phase buffer to give a final concentration of 2ng/20 µl of each monoamine/metabolite and internal standard.

Homogenising buffer

Homogenising buffer consisted of mobile phase buffer spiked with N-methyl-5-HT (internal standard). 1:100 dilution of N-methyl-5-HT 200ng/20 µl standard, made in mobile phase buffer.
Appendix B  Supplementary Data
B.1 Supplementary results for Chapter 4

Figure B.1: Effect of SNL surgery on % withdrawal response to mechanical stimulation with 4 g von Frey filament. Overall Friedman’s $\chi^2_{(15)} = 102.1$, $p < 0.001$. Within-group Friedman’s for sham $\chi^2_{(7)} = 29.7$, $p < 0.001$; for SNL $\chi^2_{(7)} = 37.2$, $p < 0.001$. % response increased vs. baseline (**$p < 0.01$ sham, #*$p < 0.05$ SNL, Wilcoxon tests) and in SNL vs. sham post surgery (**$p < 0.01$, Mann-Whitney tests). Data are shown as means ± SEM. $n = 11 – 12$.

Figure B.2: Effect of SNL surgery on mechanical response threshold in ipsilateral vs. contralateral paws. Overall Friedman’s $\chi^2_{(15)} = 121.3$, $p < 0.001$. Within-group Friedman’s for ipsi $\chi^2_{(7)} = 29.3$, $p < 0.001$; for contra: $\chi^2_{(7)} = 30.5$, $p < 0.001$. Threshold decreased vs. baseline in both paws (***$p < 0.01$ contra, ##$p < 0.01$ ipsi, Wilcoxon tests) and in ipsi vs. contra post surgery (***$p < 0.01$, Mann-Whitney tests). Data are shown as means ± SEM. $n = 11$.

Figure B.3: Effect of SNL surgery on mechanical response threshold in contralateral paws. Overall Friedman’s $\chi^2_{(15)} = 55.2$, $p < 0.001$. Within-group Friedman’s for sham $\chi^2_{(7)} = 29.8$, $p < 0.001$, for SNL $\chi^2_{(7)} = 30.5$, $p < 0.001$. Threshold decreased vs. baseline in both paws (+$p < 0.05$ contra, #*$p < 0.05$ ipsi, Wilcoxon tests) but there were no differences between sham and SNL groups. Data are shown as means ± SEM. $n = 11 – 12$.

Figure B.4: Effect of sham surgery on mechanical response threshold in ipsilateral vs. contralateral paws. Overall Friedman’s $\chi^2_{(15)} = 73.5$, $p < 0.001$. Within-group Friedman’s for ipsi $\chi^2_{(7)} = 30.9$, $p < 0.001$; for contra $\chi^2_{(7)} = 29.8$, $p < 0.001$. Threshold decreased vs. baseline in both paws (***$p < 0.01$ contra, #*$p < 0.05$ ipsi, Wilcoxon tests), and on day 13 response threshold was lowered on the ipsi compared with the contra side. Data are shown as means ± SEM. $n = 12$. 
APPENDIX B. SUPPLEMENTARY DATA

Figure B.5: Effect of SNL surgery on withdrawal latency in ipsilateral vs. contralateral paws. Two-way repeated-measures ANOVA revealed an overall effect of time ($F_{(2,40)} = 3.8, p = 0.031$), group (paw) ($F_{(1,20)} = 3.4, p = 0.045$) and time × group interaction ($F_{(2,40)} = 14.3, p = 0.001$). Withdrawal latency decreased in the ipsilateral paw compared with baseline ($# p < 0.05$) and compared with the contralateral paw post surgery ($^* p < 0.05$, **$p < 0.01$). Data are shown as means ± SEM. $n = 11 – 12$.

Figure B.6: Effect of SNL surgery on withdrawal latency in contralateral paws. Two-way repeated-measures ANOVA revealed no main effects of time, surgery or time × surgery interaction. Data are shown as means ± SEM. $n = 11 – 12$.

Figure B.7: Effect of sham surgery on withdrawal latency in ipsilateral and contralateral paws. Two-way repeated-measures ANOVA revealed no main effects of time, paw or time × paw interaction. Data are shown as means ± SEM. $n = 11 – 12$.

Figure B.8: Effect of SNL surgery on the total number of responses to acetone in the ipsilateral vs. contralateral paws. Two-way repeated-measures ANOVA revealed an overall effect of time ($F_{(2,40)} = 40.9, p < 0.001$), and effects of group (paw) ($F_{(1,20)} = 14.1, p = 0.01$) and of time × group interaction ($F_{(2,40)} = 17.0, p < 0.001$). The number of responses increased significantly post surgery compared with baseline in the ipsi paw ($+++ p < 0.001$) and in the contra paw on day 12 compared with baseline ($# p < 0.05$). The number of responses was greater in the ipsi paw than in the contra paw at all post-surgery time points ($** p < 0.01$). Data are shown as means ± SEM. $n = 11$.
Figure B.9: Effect of SNL surgery on the total number of responses to acetone in the contralateral paws. Two-way repeated-measures ANOVA showed an overall effect of time ($F_{(2,42)} = 24.0$, $p < 0.001$), but no effect of surgery or interaction. The number of responses increased significantly post surgery at all time-points compared with baseline in the sham group ($++ p < 0.01$). There was also an increase in the number of responses in the sham group on day 12 compared with baseline ($## p < 0.01$). Data are shown as means ± SEM. $n = 11 − 12$.

Figure B.10: Effect of sham surgery on the total number of responses to acetone in ipsilateral and contralateral paws. Two-way repeated-measures ANOVA showed an overall effect of time ($F_{(2,44)} = 24.0$, $p < 0.001$), but no effect of group (paw) or interaction. The number of responses increased significantly post surgery at all time-points compared with baseline in the ipsilateral paw ($++ p < 0.01$) and in the contralateral paw ($# p < 0.05$). The number of responses was greater in the ipsi paw than in the contra paw on day 4 post surgery. Data are shown as means ± SEM. $n = 12$. 
Figure B.11: Effect of CFA injection on % withdrawal response to mechanical stimulation with 4 g von Frey filament. Overall Friedman’s $\chi^2_{(15)} = 98.6, p < 0.001$. Within-group Friedman’s for control vs. CFA: $\chi^2_{(7)} = 33.2, p < 0.001$. % response increased in CFA group vs. baseline from day 3 on (## $p < 0.01$, Wilcoxon tests) and in the control group on day 11 only (+ $p < 0.05$). % response also increase in CFA group vs. control group post injection (** $p < 0.01$, *** $p < 0.001$, Mann-Whitney tests). Data are shown as means ± SEM. n = 9.

Figure B.12: Effect of CFA injection on mechanical response threshold in ipsilateral and contralateral paws. Overall Friedman’s $\chi^2_{(15)} = 119.3, p < 0.001$. Within-group Friedman’s for contralateral paw: $\chi^2_{(7)} = 37.5, p < 0.001$; for ipsilateral paw: $\chi^2_{(7)} = 34.3, p < 0.001$. Threshold decreased in both ipsilateral and contralateral paws vs. baseline (++ $p < 0.01$, # $p < 0.05$, Wilcoxon tests). Threshold also decreased in ipsilateral vs. contralateral post-pain injection (** $p < 0.01$, *** $p < 0.001$, Mann-Whitney tests). Data are shown as means ± SEM. n = 9.

Figure B.13: Effect of CFA injection on mechanical response threshold in contralateral paws. Overall Friedman’s $\chi^2_{(15)} = 106.5, p < 0.001$. Within-group Friedman’s: $\chi^2_{(7)} = 56.6, p < 0.001$. Withdrawal latency decreased in the CFA group (++ $p < 0.01$) and the control group (## $p < 0.01$) compared with baseline, and in CFA group compared with the control group post surgery (+ $p < 0.05$, ** $p < 0.01$). Data are shown as means ± SEM. n = 9.

Figure B.14: Effect of control needle insertion on mechanical response threshold in ipsilateral and contralateral paws. Overall Friedman’s $\chi^2_{(15)} = 73.8, p < 0.001$. Within-group Friedman’s: for contralateral paw: $\chi^2_{(7)} = 31.2, p < 0.001$; for ipsilateral paw: $\chi^2_{(7)} = 39.5, p < 0.001$. Threshold decreased in CFA and control groups vs. baseline (++ $p < 0.01$, # $p < 0.05$, Wilcoxon tests). There were no differences in threshold between ipsilateral and contralateral paws at any time point. Data are shown as means ± SEM. n = 9.
Figure B.15: Effect of CFA injection on withdrawal latency in ipsilateral vs. contralateral paws. Two-way repeated-measures ANOVA revealed an overall effect of time ($F_{(2,32)} = 25.5$, $p < 0.001$), paw ($F_{(1,16)} = 19.5$, $p < 0.001$) and time $\times$ paw interaction ($F_{(2,32)} = 7.3$, $p = 0.003$). Withdrawal latency decreased in the ipsilateral paw compared with baseline ($+++p < 0.001$) and compared with the contralateral paw post injection ($***p < 0.001$). Data are shown as means $\pm$ SEM. $n = 9$.

Figure B.16: Effect of CFA injection on withdrawal latency in contralateral paws. Two-way repeated-measures ANOVA revealed an overall effect of time ($F_{(2,32)} = 5.5$, $p = 0.009$), but no effect of group or time $\times$ group interaction. Withdrawal latency decreased in the control group compared with baseline on day 8 ($#p < 0.001$). Data are shown as means $\pm$ SEM. $n = 9$.

Figure B.17: Effect of control needle insertion on withdrawal latency in ipsilateral vs. contralateral paws. Two-way repeated-measures ANOVA revealed an overall effect of time ($F_{(2,32)} = 6.2$, $p = 0.005$), but no effect of treatment or time $\times$ treatment interaction. Withdrawal latency decreased in the ipsilateral paw ($^+p < 0.05$) and the contralateral paw ($^#p < 0.05$) on day 8 compared with baseline. Data are shown as means $\pm$ SEM. $n = 9$.

Figure B.18: Effect of CFA injection on the number of responses in ipsilateral vs. contralateral paws. Overall Friedman’s $\chi^2_{(5)} = 26.7$, $p < 0.001$. Within-group Friedman’s for contra vs. ipsi $\chi^2_{(2)} = 0.67$, $p = 0.013$. Withdrawal latency decreased in the ipsilateral paw compared with baseline ($^+p < 0.05$) and compared with the contralateral paw post injection ($**p < 0.01$). Data are shown as means $\pm$ SEM. $n = 9$. 
Figure B.19: Effect of CFA injection on withdrawal latency in contralateral paws. Two-way repeated-measures ANOVA revealed no main effects of time, treatment or time × treatment interaction. Data are shown as means ± SEM. n = 9.

Figure B.20: Effect of control needle insertion on the number of responses in ipsilateral vs. contralateral paws. Two-way repeated-measures ANOVA revealed an overall effect of time ($F(2,32) = 4.9, p = 0.014$), but no effect of paw or time × paw interaction. There were no significant post hoc comparisons. Data are shown as means ± SEM. n = 9.
B.2 Supplementary results for Chapter 5

Figure B.21: Effect of SNL surgery on % withdrawal response to mechanical stimulation with 4 g von Frey filament. Overall Friedman’s $\chi^2_{(17)} = 82.9$, $p < 0.001$. Within-group Friedman’s: not significant for sham; for SNL $\chi^2_{(8)} = 35.2$, $p < 0.001$. % response increased In SNL vs. baseline (***$p < 0.05$ SNL, Wilcoxon tests) and in SNL vs. sham post surgery (***$p < 0.01$, *$p < 0.05$, Mann-Whitney tests). Data are shown as means ± SEM. $n = 11 – 12$.

Figure B.22: Effect of SNL surgery on mechanical response threshold in ipsilateral vs. contralateral paws. Overall Friedman’s $\chi^2_{(17)} = 147.0$, $p < 0.001$. Within-group Friedman’s for ipsi: $\chi^2_{(8)} = 43.2$, $p < 0.001$; for contra: $\chi^2_{(8)} = 52.6$, $p < 0.001$. Threshold decreased vs. baseline in both paws (++$p < 0.01$ contra, ##$p < 0.01$ ipsi, Wilcoxon tests) and in ipsi vs. contra post surgery (**$p < 0.01$, Mann-Whitney tests). Data are shown as means ± SEM. $n = 11$.

Figure B.23: Effect of SNL surgery on mechanical response threshold in contralateral paws. Overall Friedman’s $\chi^2_{(17)} = 78.2$, $p < 0.001$. Within-group Friedman’s for sham $\chi^2_{(8)} = 49.2$, $p < 0.001$, for SNL $\chi^2_{(8)} = 50.1$, $p < 0.001$. Threshold decreased vs. baseline in both paws (++$p < 0.01$ sham, #p < 0.05 SNL, Wilcoxon tests) but there were no differences between sham and SNL groups. Data are shown as means ± SEM. $n = 11 – 12$.

Figure B.24: Effect of sham surgery on mechanical response threshold in ipsilateral vs. contralateral paws. Overall Friedman’s $\chi^2_{(17)} = 90.1$, $p < 0.001$. Within-group Friedman’s for ipsi $\chi^2_{(8)} = 36.1$, $p < 0.001$; for contra $\chi^2_{(8)} = 49.2$, $p < 0.001$. Threshold decreased vs. baseline in both paws (++$p < 0.01$ contra from day 1, #p < 0.05 ipsi from day 3, Wilcoxon tests). Data are shown as means ± SEM. $n = 12$. 
Figure B.25: Effect of SNL surgery on withdrawal latency in ipsilateral vs. contralateral paws. Two-way repeated-measures ANOVA revealed an overall effect of time ($F_{(3,54)} = 22.6, p < 0.001$) and group (paw) ($F_{(1,18)} = 15.4, p = 0.001$). Withdrawal latency decreased in ipsilateral and contralateral paws compared with baseline (+++p < 0.001 ipsi, #p < 0.05 contra), and ipsilateral was lower than contralateral on days 8 and 63/65 post surgery (**p < 0.01). Data are shown as means ± SEM. n = 10.

Figure B.26: Effect of SNL surgery on withdrawal latency in contralateral paws. Two-way repeated-measures ANOVA revealed a main effect of time ($F_{(3,60)} = 8.4, p < 0.001$), but no effect of surgery or time × surgery interaction. Withdrawal latency decreased in the sham group on day 16 (+p < 0.05) and in SNL group on days 8 and 16 (#p < 0.05) post surgery compared with baseline. Data are shown as means ± SEM. n = 10−12.

Figure B.27: Effect of sham surgery on withdrawal latency in ipsilateral and contralateral paws. Overall Friedman’s analysis revealed no significant effect. Data are shown as means ± SEM. n = 12.

Figure B.28: Effect of SNL surgery on the total number of responses to acetone in the ipsilateral vs. contralateral paws. Overall Friedman’s analysis: $\chi^2_{(7)} = 59.9, p < 0.001$. Within-group Friedman’s showed an effect of time in the ipsilateral paw. The number of responses increased significantly post surgery compared with baseline in the ipsi paw (++p < 0.01). The number of responses was greater in the ipsi paw than in the contra paw at all post-surgery time points (**p < 0.001, ***p < 0.01). Data are shown as means ± SEM. n = 11.
Figure B.29: Effect of SNL surgery on the total number of responses to acetone in the contralateral paws. Overall Friedman’s analysis: $\chi^2(7) = 16.3$, $p = 0.023$. Within-group Friedman’s: for sham $\chi^2(3) = 8.9$, $p = 0.031$, not significant for SNL. The number of responses increased on days 4 and 12 post surgery compared with baseline in the sham group (*$p < 0.05$). Data are shown as means ± SEM. $n = 11 - 12$.

Figure B.30: Effect of sham surgery on the total number of responses to acetone in ipsilateral and contralateral paws. Overall Friedman’s analysis: $\chi^2(7) = 41.6$, $p < 0.001$. Within-groups Friedman’s: for ipsi, $\chi^2(3) = 26.0$, $p < 0.001$; for contralateral, $\chi^2(3) = 8.3$, $p < 0.031$ The number of responses increased significantly post surgery compared with baseline in the ipsilateral paw (**$p < 0.05$) and in the contralateral paw on days 4 and 12 post surgery (†$p < 0.05$). Data are shown as means ± SEM. $n = 12$.

Figure B.31: Latency to get onto the platform in the acquisition phase of the Morris water maze. Two-way repeated-measures ANOVA of log-transformed data revealed a significant effect of time ($F_{(4,84)} = 15.9$, $p < 0.001$) but no effect of surgery or interaction. Latency to the platform decreased over time in both sham and SNL groups (LSD post hoc analysis: *$p < 0.05$ for SNL days 2–5 vs. day 1, **$p < 0.01$ for sham days 3–5 vs. day 1). Data are shown as means ± SEM. $n = 11 - 12$.

Figure B.32: Average swim speed in the acquisition phase of the Morris water maze. Two-way repeated-measures ANOVA revealed a significant effect of time ($F_{(4,84)} = 19.7$, $p < 0.001$) but no effect of surgery or interaction. Average swim speed decreased over time in both sham and SNL groups (LSD post hoc analysis: *$p < 0.05$ for SNL days 2–5 vs. day 1, **$p < 0.05$ for sham days 2–5 vs. day 1). The SNLs had significantly lower swim speed than the shams on acquisition day 3 (Φ$p < 0.05$). Data are shown as means ± SEM. $n = 11 - 12$. 
Figure B.33: % time in quadrants and platform zones in the forward probe trial of the Morris water maze. Unpaired two-tailed t-tests revealed no differences between sham and SNL groups. As expected, the % time spent in the SW quadrant (where the platform had been located) was above the level of chance (25%). Data are shown as means ± SEM. n = 11 – 12.

Figure B.34: % time spent in the SW quadrant in trial 1 of reversal training in the Morris water maze. Two-way repeated-measures ANOVA revealed an overall effect of time (F(4,84) = 2.96, p = 0.024). There was an overall effect of surgery (F(1,21) = 2.17, p = 0.08). The % time spent in the SW quadrant during trial 1 decreased in the SNL group on day 5 compared with day 1 (#p < 0.05). SNL rats spent a significantly greater % of time in the SW quadrant than sham controls during trial 1 on day 3 (**p < 0.01). Data are shown as means ± SEM. n = 11 – 12.

Figure B.35: % time spent in NE quadrant in trial 1 of reversal training. Two-way repeated ANOVA revealed no significant overall effects. Data are shown as means ± SEM. n = 11 – 12.

Figure B.36: Average swim speed in reversal training in the Morris water maze. Two-way repeated-measures ANOVA revealed an overall effect of time (F(4,84) = 20.7, p = < 0.001). There was a decrease in the swim speed over time in both sham and SNL groups (sham: *p < 0.05 on days 2–5 vs. day 1; SNL: #p < 0.05 on days 3–5 vs. day 1). The SNL rats had a significantly lower swim speed than sham controls on day 4 (**p < 0.05).
Figure B.37: % time spent in quadrants and platform zones in the reversal probe trial of the Morris water maze. Unpaired two-tailed t-test showed that SNL rats spent significantly less time in the NE quadrant than sham controls (*p < 0.05).
B.3 Supplementary results for Chapter 6

**Figure B.38:** Effect of treatment on % withdrawal response to 4 g von Frey filament in ipsilateral paws. Overall Friedman’s ANOVA revealed a significant effect ($\chi^2_{(19)} = 82.6$, $p < 0.001$). There were significant effects of time in all groups except the sham-water group ($p < 0.01$). Kruskal-Wallis tests revealed an effect of group from week 3 post surgery on ($p < 0.01$). % response increased post surgery compared with baseline in sham-ami ($^\#p < 0.05$), SNL-ami ($^\#p < 0.05$) and SNL-water ($^\#p < 0.05$) groups (Wilcoxon test). The % response was higher in the SNL-water group that in the sham-water group on weeks 3–9 ($^***p < 0.001$, $^**p < 0.01$, $^*p < 0.05$) and in the SNL-ami vs. sham-ami groups on weeks 1, 6 and 9 ($^p < 0.05$). % response also increased in sham-ami compared with sham-water ($^\#^\#p < 0.01$, $^\#p < 0.05$). Data are shown as means ± SEM. $n = 8–9$.

**Figure B.39:** Effect of treatment on mechanical response threshold in the contralateral paws. An overall Friedman’s ANOVA by ranks revealed a significant effect ($\chi^2_{(19)} = 65.4$, $p < 0.001$). There were significant effects of time in all four treatment groups ($p < 0.01$). A Kruskal-Wallis test showed an effect of group at week 9, but there were no valid significant comparisons. Data are shown as means ± SEM. $n = 8–9$.

**Figure B.40:** Effect of treatment on paw withdrawal latency in the contralateral paws. An overall Friedman’s ANOVA by ranks did not reveal a significant effect, and therefore post hoc tests were not justified. Data are shown as means ± SEM. $n = 8–9$.

**Figure B.41:** Effect of treatment on total number of paw withdrawals following acetone application. Overall Friedman’s revealed a significant effect ($\chi^2_{(31)} = 73.0$, $p < 0.001$). There were significant effects of time in all four treatment groups. % response increased post surgery compared with baseline in sham-ami ($^\#^\#p < 0.01$, $^\#p < 0.05$), SNL-ami ($^\#p < 0.05$), SNL-ami ($^\#p < 0.05$) and SNL-water ($^\#p < 0.05$) groups (Wilcoxon tests). Kruskal-Wallis tests revealed an effect of group at week 2 ($\chi^2_{(31)} = 7.8$, $p = 0.05$), and at this time point the number of responses was significantly higher in the SNL-ami group than in the SNL-water group ($^p < 0.05$). Data are shown as means ± SEM. $n = 8–9$. 
Figure B.42: Latency to get onto the platform in the acquisition phase in the Morris water maze. Overall Friedman’s tests revealed a significant effect ($p < 0.001, \chi^2_{(19)} = 51.3$), and Friedman’s tests also showed significant effects of time in sham-water and sham-ami groups ($p < 0.05$), but not SNL-water or SNL-ami groups. Latency decreased significantly over time in the sham-water group ($*p < 0.05$, days 2–5 vs. day 1) and sham-ami group ($\#p < 0.05$, days 3–5 vs. day 1). Latency decreased in the SNL-water group on day 5 only compared with day 1 ($\alpha p < 0.05$) and in the SNL-ami on day 4 only ($\beta p < 0.05$). Data are shown as means $\pm$ SEM. $n = 8 – 9$.

Figure B.43: Average swim speed in the acquisition phase of the Morris water maze. Overall Friedman’s tests revealed a significant effect ($p \lesssim 0.05, \chi^2_{(19)}$), and Friedman’s tests showed significant effects of time in sham-water and SNL-ami groups ($p < 0.05$), but not in SNL-water or sham-ami groups. Swim speed was increased significantly on day 4 compared with day 1 in the sham-ami group ($*p < 0.05$) and decreased on days 2 and 5 compared with day 1 in the SNL-ami group ($\#p < 0.05$). Kruskal-Wallis tests revealed a significant effect of group on days 4 and 5. Higher swim speeds were observed in the sham-ami group than in the sham-water group on days 4 and 5 ($\alpha p < 0.01$, $\alpha\alpha p < 0.01$). The sham-ami group were also faster than the SNL-ami (days 3–5, $\alpha p < 0.05$, $\alpha\alpha p < 0.01$) and the SNL-water (days 3 and 4, $\delta p < 0.05$, $\delta\delta p < 0.01$) groups. Data are shown as means $\pm$ SEM. $n = 8 – 9$.

Figure B.44: % time spent in the SW quadrant in reversal training in the Morris water maze. Friedman’s test revealed no overall significant difference; however, there was a trend for a decrease over time in the SNL-water group. Kruskal-Wallis tests did not reveal any group effects at any of the time points. Data are shown as means $\pm$ SEM. $n = 8 – 9$.

Figure B.45: % time spent in each quadrant during the reversal probe trial in the Morris water maze. Two-way ANOVA revealed no significant main effects in the quadrants of interest (SW and NE). Data are shown as means $\pm$ SEM. $n = 8 – 9$. 

388
Appendix C  Ethovision® System
C.1 Ethovision® 3.1 Quick Start Guide

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APPENDIX C. ETHOVISION® SYSTEM

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C.2 Ethovision® XT Novel Object Test

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C.3 Ethovision® XT Morris Water Maze

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D.1 Using ImageJ for western immunoblotting

The National Institutes of Health (NIH) version of ImageJ software was used for analysis of western immunoblot images. The programme was downloaded from the website: http://rsbweb.nih.gov/ij/download.html The western immunoblot to be analysed for the protein of interest is opened through ImageJ. The rectangular drawing tool (yellow) is used to map the area around the band of interest (Figure D.1).

Figure D.1
In the Analyze drop-down menu, the Set Measurements item is selected. The measurements required are Area and Integrated density (Figure D.2).

![ImageJ Set Measurements](image)

**Figure D.2**

The measurements are calculated by selecting Measure in the Analyze drop-down menu and the results are presented in a separate window.

A rectangle of equal area is drawn directly above the band to measure the background integrated density and this is subtracted from the integrated density calculated for the band to give a corrected value.

The same measurement is performed for the loading control (β-actin), and the ratio of the protein of interest to β-actin is used a measure of relative protein expression.
D.2 Using ImageJ for immunohistochemistry

The McMaster Biophotonics Facility (MBF) version of ImageJ software was used for the analysis of immunohistochemically-stained compressed Z-stack images. The program was downloaded from http://www.macbiophotonics.ca/downloads.htm. The confocal image to be analysed is opened through ImageJ. Areas of synaptophysin expression are indicated by green fluorescence (AF488-coupled secondary antibody); see Figure D.3. These areas are converted to black and white images using the following processes:

In the **Image** drop-down menu, select **Type** → 8-bit

In the **Process** drop-down menu, select **Binary** → Make Binary

In the **Process** drop-down menu, select **Enhance contrast**, change to 0.6%.

![ImageJ process steps](image.png)

**Figure D.3**

In the **Analyze** drop-down menu, the **Set measurements** item is selected. The measurements required are **Area** and **Area Fraction**.

The measurements are calculated by selecting **Measure** in the **Analyze** drop-down menu and the results are presented in a separate window.
Macros

For the processing of large numbers of images it is useful to create a *macro*, or short sequence of programming language. This records the sequence of steps in the image analysis and can be saved for subsequent use.

To create a macro in ImageJ:

Open image of interest in ImageJ.

From the **Plugins** drop-down menu, select **Macros → Record**.

Repeat steps as described above; once finished, select **Create** from the macro window (Figure D.4) and save the resulting file.

![ImageJ Macro Recorder](image)

**Figure D.4**

For subsequent images:

Open image in ImageJ.

From **Macros** in the **Plugins** section, select **Run** and choose the saved macro from the browser window.
Batch processing

Large numbers of images may be processed more efficiently using the batch mode function of ImageJ. Images to be analysed must be stored in the same file location. To run a macro in batch mode:

Select **Process, Batch → Macro**

Select macro to be used.

Create an input folder with your images to be analysed and an empty output folder where processed binary images are to be stored (Figure D.5).

Click **Process** and the results for the series of images in the input folder appear in a separate results window.

![ImageJ Macro Dialog](image)

**Figure D.5**

The area fraction indicates the % of the total tissue that is occupied by synaptophysin-positive staining and is calculated for each section from each rat.
Appendix E  Clinical Study Materials
E.1 Patient questionnaire

A Neuropsychological Study of Pain and Cognitive Function

Name: ____________________________________________

Age: ____________________________________________

Address: _________________________________________
__________________
__________________
__________________

Contact Tel. No.: ________________________________

Email: _________________________________________
CONSENT FORM

I _________________________________________________ (Full name)
of ________________________________________________ (Address)

hereby fully and freely consent to participate in the above mentioned study, which investigates aspects of cognitive brain functioning in chronic pain.

I understand that I may withdraw my consent at any stage during the study.
I acknowledge the purpose of the study and any risks involved in the study procedures.
I understand that the study consists of:

a. A pain questionnaire
b. A depression questionnaire
c. An anxiety questionnaire
d. A psychometric assessment

The nature and purpose of such procedures have been described to me in the Information Sheet and have been explained to me by:
__________________________________________________ (name of researcher)
and I have discussed these matters with him/her to my satisfaction.

Signature of participant: ___________________________
Witness: ___________________________
Date: ___________________________

Declaration by the investigator

I confirm that I have provided an Information Sheet and explained the nature and effect of the procedures to the participant and that his/her consent has been given freely and voluntarily.

Signed:____________________ Date:_______________

Test subject consent form.
APPENDIX E. CLINICAL STUDY MATERIALS

For each of the following questions, please circle your answer.

Have you ever been diagnosed with cognitive impairment?
(e.g. a learning disability or dementia including Alzheimer’s disease)  Yes / No

Have you ever suffered a traumatic head injury?  Yes / No

Do you regularly abuse alcohol, prescription drugs or other substances?  Yes / No

Do you suffer from:

Any major psychiatric illness?
(e.g. Major Depressive Disorder, Generalized Anxiety Disorder, Schizophrenia, Autism Spectrum Disorder)  Yes / No

Diabetes?  Yes / No

Epilepsy or seizures?  Yes / No

If you answered yes to any of the above you are not eligible to take part in this study. Thank you for your time.
Date of Birth: ___________________________

Gender, please circle:
Male  /  Female

Number of years of education: ____________________

Please include primary, secondary and third level education. Note: In the current Irish education system primary schooling is 8 years duration and secondary schooling is 5 years (or 6 including transition year).

A number of common lifestyle factors can affect the types of cognition we will measure today. Please answer the following where applicable:

Do you currently smoke cigarettes?  
Yes  /  No

If so, please indicate the time you smoked your most recent cigarette?
(If you are currently using nicotine replacement therapy, please indicate the time the treatment was most recently used)
__________________________________________________________

When did you last consume an alcoholic drink?
_____________________________________________________

When did you last consume a caffeine-containing beverage? (e.g. coffee, tea, energy drinks such as Coca-Cola®, Red Bull® or Lucozade®)
_____________________________________________________

Subject code: _________
1. Currently (i.e. over the past few months) have you been troubled by pain or discomfort, either all the time, or on and off?

   Yes / No

   If you answered no to question 1, you may proceed to page 7.

2. Has this pain lasted for more than 3 months?

   Yes / No

   If you answered no to question 2, you may proceed page 7.

3. On the diagram, shade in the areas where you feel pain. Put an X on the area that hurts the most.

4. Number of months since diagnosis of your pain: _____________________

5. What treatments or medications are you receiving for your pain?

   __________________________
   __________________________
   __________________________

Page 3 of Patient Questionnaire
6. In the past 24 hours, how much relief have pain treatments or medications provided? Please circle the one percentage that most shows how much relief you have received.

0%  10%  20%  30%  40%  50%  60%  70%  80%  90%  100%

No relief  Complete relief

7. In your daily life, do you ever experience difficulty with the following?

(i) Concentrating: Yes / No

(ii) Remembering items over a short period of time:
    (for example phone numbers etc.): Yes / No

(iii) Solving problems or making decisions?: Yes / No

8. If yes, do you think your pain contributes to these difficulties, please circle:

Yes / No

Subject code: _________
For the following questions, please **circle** the number that best describes your pain

1. How would you rate your pain, on a 0-10 scale, at its **worst** in the last 24 hours, where 0 is 'No Pain' and 10 is 'Pain as bad as it could be'?

   | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
---|---|---|---|---|---|---|---|---|---|---|----|
   | No pain | Pain as bad as it could be |

2. How would you rate your pain, on a 0-10 scale, at its **least** in the last 24 hours?

   | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
---|---|---|---|---|---|---|---|---|---|---|----|
   | No pain | Pain as bad as it could be |

3. How would you rate your pain on a 0-10 scale at the **present time**, that is, right now?

   | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
---|---|---|---|---|---|---|---|---|---|---|----|
   | No pain | Pain as bad as it could be |

4. In the past 3 months, how intense was your **worst pain**?

   | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
---|---|---|---|---|---|---|---|---|---|---|----|
   | No pain | Pain as bad as it could be |

5. In the past 3 months, **on average**, how intense was your pain?

   | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
---|---|---|---|---|---|---|---|---|---|---|----|
   | No pain | Pain as bad as it could be |

6. About how many days in the past three months have you been kept from your usual activities (work, school or housework) because of pain? **Place a tick in the appropriate box**

   - 0-6 days
   - 7-14 days
   - 15-30 days
   - 31 or more days
7. In the past 3 months how much has pain interfered with your daily activities where 0 is “no interference” and 10 is “unable to carry on any activities”

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<tbody>
<tr>
<td>No interference</td>
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<td>Unable to carry on any activities</td>
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8. In the past 3 months how much has pain interfered with your ability to take part in recreational, social and family activities?

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<tr>
<td>No interference</td>
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<td>Unable to take part in recreational or social activities</td>
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9. In the past three months, how much has pain interfered with your ability to work (including college, housework)?

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<tr>
<td>No interference</td>
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<tr>
<td>Extreme interference</td>
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10. In the past three months, how much has pain interfered with your concentration, memory, problem solving or decision making?

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</tr>
</thead>
<tbody>
<tr>
<td>No interference</td>
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<tr>
<td>Extreme interference</td>
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E.2 Test forms

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E.3 Invitation letter for patient volunteers

Dear Ms/Mrs/Mr ______________________,

The Schools of Psychology and Medicine, National University of Ireland Galway, are currently undertaking research into the psychological effects of chronic pain. We are contacting you to let you know more about the study and to see if you might be interested in taking part.

The study seeks to find out if chronic pain is associated with changes in cognitive functioning, which includes processes such as learning, memory, attention and problem-solving. Enclosed is an information sheet which explains what is involved.

Unfortunately, people with a pre-existing learning disability, psychiatric illness, history of traumatic head injury or substance abuse, or diabetes are not eligible to take part in this study. If you think you are eligible and might like to take part, we invite you to contact our researcher, Orla Moriarty (telephone and email contact details are provided below and on the information sheet), who can tell you a little more about the study and answer any questions you may have. By contacting us, you are not committing yourself to participating in the study, but you are giving permission to talk to you in more detail about the study.

If you decide to take part you will be given a time and date to meet with a member of the research team. We would also ask you to bring along a list of medications you are currently taking. In addition, if you wear glasses or contact lenses to correct for visual impairment, we would ask you to bring these with you. You will be offered compensation to the value of €30 for your time.

Thank you for taking the time to consider this letter.

Yours sincerely,

Dr. Brian McGuire,
Project Supervisor
School of Psychology,
National University of Ireland, Galway.
Clinical Psychologist,
Pain Clinic, Galway University Hospital.

Letter inviting patients to participate in the study, page 1.
Researcher Contact Details:
Orla Moriarty,  
PhD Researcher,  
Pharmacology and Therapeutics,  
School of Medicine,  
National University of Ireland, Galway,  
University Road,  
Galway,  
Tel: 0863918862 or (091) 493272  
Email: o.moriarty1@nuigalway.ie
E.4 Advertisement for healthy volunteers

The schools of Psychology and Medicine, NUIG require:

Healthy Volunteers to Participate in a Research Study

This study seeks to find out more about the effects of chronic pain on cognitive functions like memory and attention. Healthy volunteers will be asked to complete some questionnaires and to do some simple tests that examine memory, attention and other functions. The process will take about an hour and a half and participants will be offered €30 as compensation for their time. In order to participate in this study, you must fulfil the following criteria:

- Be over 18 years of age
- Have NO history of chronic pain, brain injury, psychiatric illness, substance abuse, epilepsy or seizures, or diabetes
- Have NO history of cognitive impairment (such as a learning disability, a brain injury or dementia)
- Have sufficient English language ability to complete the tests

For further information, please contact Orla Moriarty, Pharmacology and Therapeutics, School of Medicine, NUIG Galway, Tel: 086 3918862/091 493272, email: o.moriarty1@nuigalway.ie, or project supervisor Dr. Brian McGuire, School of Psychology, NUIG Galway and Clinical Psychologist, Pain Clinic, Galway University Hospital, Tel: 091 492954, email: brian.mcguire@nuigalway.ie.

Figure E.1: Advertisement inviting members of the public to participate as controls in the study.
E.5 Information sheet for patient volunteers

INFORMATION SHEET FOR PATIENT VOLUNTEERS

A Neuropsychological Study of Pain and Cognitive Function

You are invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. This information sheet will tell you about it. If you agree to take part, we will ask that you sign a Consent Form. If there is anything that you are unsure about we will be happy to explain it to you. Please take as much time as you need to read this information sheet. You should only consent to participate when you feel that you understand what is being asked of you, and that you have had enough time to think about your decision.

Purpose of the study
This study seeks to investigate the impact of chronic pain on cognitive functions like memory, attention and problem-solving. The overall goal is to better understand the consequences of chronic pain and so to improve future treatments and interventions. In order to properly investigate the effects of chronic pain, it is necessary to compare chronic pain patients with people who are not in pain, and this is why you have been asked to participate.

Am I eligible?
Patients with a diagnosis of neuropathic (nerve) pain or radiculopathy (pain radiating from one part of the body to another) are invited to participate. Unfortunately, patients under the age of 18, patients with pre-existing cognitive impairment (including a learning disability or dementia), patients who have suffered from a traumatic head injury, patients who regularly abuse alcohol or other substances, patients with a psychiatric illness (such as schizophrenia, depression, bipolar disorder or generalised anxiety disorder), patients suffering from epilepsy or recurring seizures and patients with diabetes are not eligible to take part in this study. If you are unsure as to your eligibility, please ask a member of the research team.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you can keep this Information Sheet and you can contact a member of the research team with any other questions you may have. If you do decide to take part, you are free to withdraw at any time and without giving reason. A decision to withdraw at any time, or a decision not to take part, will not affect your rights in any way and will not affect the care you are receiving. By completing the testing, you are consenting to be involved in the research anonymously.

What will happen to me if I take part?
If you are eligible and decide to take part, you will be given a date and time to meet a member of the research team, who will explain the tests to you in detail, and gain your written consent to participate in a psychometric assessment. This involves various tests that measure intelligence, memory, attention and more complex executive functions.
functions or problem-solving abilities. These tests will give us an indication of your cognitive functioning and should last for about 1 and a half hours. You will also be asked to complete a short pain questionnaire and a questionnaire that measures anxiety at the time of testing. Some of the tests/questionnaires will be ‘pen and paper-based’ and some will be done on a computer. On completion of the tests you will receive compensation to the value of €30 for the time spent taking part in the study.

How long will my part in the study last?
The tests are expected to last about 1 and a half hours altogether.

What are the possible advantages to taking part?
There will be no immediate benefit to you from taking part in this project but it may help us to better understand the cognitive consequences of chronic pain. In this way, we hope to improve the future treatment of, and interventions for chronic pain.

What are the possible disadvantages to taking part?
Taking part in this study poses no direct risks to your health.

What do I have to do?
You do not need to make any changes to your daily lifestyle during your participation in this study. On the day of testing, you will be asked to complete the questionnaires and the tests described above.

What happens at the end of the study?
The information from the various tests will be combined with those from other participants and analysed on a computer. Your responses are absolutely confidential and will only be used to extend our understanding of chronic pain generally. Information will be stored and handled using code numbers and not your name. We will share our findings with other scientists and we may publish our findings, but all data will be reported as group results – no individual data will be published and you will not be identifiable in any reports.

What if I have a complaint during my participation in the study?
The research team will be available for you to contact if you have any complaints or queries during your participation in the study.
Whom do I contact for more information or if I have further concerns?
If you have any further questions or concerns about the study, please contact:

Orla Moriarty,
PhD Researcher,
Pharmacology and Therapeutics,
School of Medicine,
National University of Ireland, Galway.
Tel.: 086 3918862 or (091) 493272
Email: o.moriarty1@nuigalway.ie

Dr Brian McGuire,
Project Supervisor,
School of Psychology,
National University of Ireland, Galway.
Tel. (091) 493266
Email: brian.mcguire@nuigalway.ie

If you have concerns about this study and wish to contact someone independent and in confidence, you may contact The Chairperson of the NUI Galway Research Ethics Committee, c/o Office of the Vice-President for Research, NUI Galway, ethics@nuigalway.ie

THANK YOU FOR TAKING THE TIME TO READ THIS

Information sheet provided to patient volunteers, page 3.
E.6 Information sheet for healthy volunteers

INFORMATION SHEET FOR HEALTHY VOLUNTEERS

A Neuropsychological Study of Pain and Cognitive Function

You are invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. This information sheet will tell you about it. If you agree to take part, we will ask that you sign a Consent Form. If there is anything that you are unsure about we will be happy to explain it to you. Please take as much time as you need to read this information sheet. You should only consent to participate when you feel that you understand what is being asked of you, and that you have had enough time to think about your decision.

Purpose of the study
This study seeks to investigate the impact of chronic pain on cognitive functions like memory, attention and problem-solving. The overall goal is to better understand the consequences of chronic pain and so to improve future treatments and interventions. In order to properly investigate the effects of chronic pain, it is necessary to compare chronic pain patients with people who are not in pain, and this is why you have been asked to participate.

Am I eligible?
Unfortunately, people under the age of 18, people with pre-existing cognitive impairment (including a learning disability or dementia), people who have suffered from a traumatic head injury, people who regularly abuse alcohol or other substances, people with a major psychiatric illness, people suffering from epilepsy or recurring seizures and people with diabetes are not eligible to take part in this study. If you are unsure as to your eligibility, please ask a member of the research team.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you can keep this Information Sheet and you can contact a member of the research team with any further questions you may have. If you decide to take part you are free to withdraw at any time and without giving reason. A decision to withdraw at any time, or a decision not to take part, will not affect your rights in any. By completing the testing, you are consenting to be involved in the research anonymously.

What will happen to me if I take part?
If you are eligible and decide to take part, you will be given a date and time to meet a member of the research team, who will explain the tests to you in detail, and gain your written consent to participate in a psychometric assessment. This involves various tests that measure intelligence, memory, attention and other areas of brain function. These tests will take about an hour and will give us an indication of your cognitive functioning. You will also be asked to complete some short questionnaires that measure depression, anxiety and pain at the time of testing. Some of the tests/questionnaires will be ‘pen and paper-based’ and some will be done on a
On completion of the tests, you will receive compensation to the value of €30 for the time spent taking part in the study.

**How long will my part in the study last?**
The tests are expected to last about 1 hour altogether. A suitable time and venue will be arranged with a member of the research team. In most cases testing will be carried out at the National University of Ireland, Galway. However, a researcher may visit your home to carry out testing if this is more convenient for you.

**What are the possible advantages to taking part?**
There will be no immediate benefit to you from taking part in this project but it may help us to better understand the psychological consequences of chronic pain – the effect of chronic pain on learning, memory, attention and other similar brain processes. In this way, we hope to improve the future treatment and interventions for chronic pain.

**What are the possible disadvantages to taking part?**
Taking part in this study poses no direct risks to your health.

**What do I have to do?**
You do not need to make any changes to your daily lifestyle during your participation in this study. On the day of testing, you will be asked to complete the questionnaires and the tests described above.

**What happens at the end of the study?**
The information from the various tests will be combined with those from other participants and analysed on a computer. Your responses are absolutely confidential and will only be used to extend our understanding of chronic pain generally. Information will be stored and handled using code numbers and not your name. We will share our findings with other scientists and we may publish our findings, but all data will be reported as group results – no individual data will be published and you will not be identifiable in any reports.

**What if I have a complaint during my participation in the study?**
The research team will be available for you to contact if you have any complaints or queries during your participation in the study. Alternatively you may contact the Chair of the Research Ethics Committee at NUI, Galway (see below).
Whom do I contact for more information or if I have further concerns?
If you have any further questions or concerns about the study, please contact:

Orla Moriarty, PhD Researcher, Pharmacology and Therapeutics, School of Medicine, National University of Ireland, Galway. Tel. (091) 493272 or 086 3918862 Email: o.moriarty1@nuigalway.ie

Dr Brian McGuire, Project Supervisor, School of Psychology, National University of Ireland, Galway. Tel. (091) 493266 Email: brian.mcguire@nuigalway.ie

If you have concerns about this study and wish to contact someone independent and in confidence, you may contact The Chairperson of the NUI Galway Research Ethics Committee, c/o Office of the Vice-President for Research, NUI Galway, ethics@nuigalway.ie

THANK YOU FOR TAKING THE TIME TO READ THIS