<table>
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<th><strong>Title</strong></th>
<th>Altered nociceptive responding in animal models of affective disorders: role of monoamines and immune mediators</th>
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<td><strong>Author(s)</strong></td>
<td>Burke, Nikita</td>
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1. General introduction

Depression is one of the most prevalent forms of psychiatric illness and is associated with significant disability, impaired health-related quality of life and high mortality (Cuijpers and Smit, 2002, Nestler et al., 2002, Ormel et al., 1994, Spitzer et al., 1995). Depression is the most common mental health disorder, presenting in 10–15% of patients in primary care (Ormel et al., 1994, Spitzer et al., 1995), and up to one in five individuals will suffer depression in their lifetime (Kessler et al., 2003, Andrade et al., 2003). By 2020, depression is predicted to be the second leading cause of world disability (WHO, 2001) and by 2030, it is expected to be the largest contributor to global disease burden (WHO, 2008). Depression is associated with multiple symptoms including psychological and physical complaints (DSM-5, 2013). Psychological symptoms include depressed mood, anhedonia, feelings of worthlessness or guilt, altered concentration, and suicidality. Physical symptoms of depression include altered appetite and weight, disturbed sleep, psychomotor agitation or retardation, and fatigue (DSM-5, 2013). Depression is associated with a number of comorbid psychiatric and physical illnesses including anxiety, panic disorder, cardiovascular disease, obesity, and diabetes (Noel et al., 2004, Kessler et al., 1998, Hirschfeld, 2001, Celano and Huffman, 2011, Luppino et al., 2010). Another disorder that frequently co-occurs with depression is chronic pain. In fact, the comorbidity of depression and chronic pain represents a significant health burden (Bair et al., 2003), the implications of which will be discussed further in this chapter. Physiological or nociceptive pain is necessary, protective and adaptive, allowing survival, alerting us to potential or actual tissue damage and motivating us into action to limit further injury and begin repair and recovery. Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (International Association for the Study of Pain). Noxious stimuli activate Aδ and C-fibre nociceptive primary
afferent neurons, which transmit information relating to sharp well-localised and slow aching pain, respectively, to the dorsal horn of the spinal cord (Snider and McMahon, 1998). Here, they synapse with second order neurons and ascend to the brain (Willis, 1985a, Willis, 1985b) via the ascending pain pathways that project directly to regions involved in the sensory-discriminative (location, duration, intensity and quality of pain) and the affective-motivational (emotion, unpleasant quality, learning and memory of pain) components of the pain experience. The intrinsic unpleasant and emotional quality of pain is of key significance when considering the overlap between depression and pain. Incoming pain signals may be modulated at the level of the spinal cord prior to ascending to higher brain centres such as the thalamus, somatosensory cortex, prefrontal cortex, limbic system, anterior cingulate cortex, insula, periaqueductal grey (Melzack and Wall, 1965) or regulated (inhibited or facilitated) by these higher brain regions via pathways descending directly to the dorsal horn (Fields et al., 1991, Willis, 1985b, Willis, 1985c). Under normal conditions, a balance of inhibition/facilitation occurs, ensuring that only necessary stimuli reach the level of consciousness. However, descending facilitation and/or peripheral and central sensitization (increased excitability and synaptic efficacy of nociceptor pathways) may be involved in pathological pain conditions such as chronic inflammatory, neuropathic or visceral pain (Pertovaara, 1998, Desmeules et al., 2003, Woolf, 2011). These changes contribute to increased pain perception, namely hyperalgesia, enhanced pain to a normally painful stimulus, and allodynia, pain to a normally innocuous stimulus.
Fig. 1.1 Ascending and descending pain pathways. The ventral spinothalamic pathway innervates thalamic nuclei subsequently synapsing with third-order neurons that terminate in the cortex and encodes the sensory-discriminative aspects of pain. Neurons in the spinoparabrachial pathway synapse with third-order neurons that terminate in the hypothalamus and the amygdala and are important in the affective-motivational component of pain. The descending pain pathways originate in neurons in higher brain regions such as the cortex, hypothalamus, and amygdala. Neurons from these regions project to the periaqueductal grey (PAG) and the rostroventral medulla (RVM) and finally to the dorsal horn of the spinal cord, where they modulate pain signals. Adapted from (Olango, 2012)
Pain is the most common physical symptom reported in the general population and in primary care (Kroenke, 2003, Unutzer et al., 2002). Physical injury usually dissipates when healing occurs; however, in some cases, acute pain does not abate and becomes chronic – defined as pain that lasts for more than three months (Saravanakumar, 2010). The median duration of chronic pain is 7 years, which impacts significantly on the life of an individual (Breivik et al., 2006a). Chronic pain is maladaptive, causing emotional distress and reduced quality of life. Estimates of the prevalence of chronic pain in Ireland range from 1 in 8 in the general population (Breivik et al., 2006a) to 1 in 3 in a community sample (Raftery et al., 2011).

1.1 Evidence for depression-pain comorbidity

The co-occurrence of depression and chronic pain has been examined in a number of studies. Bair et al. (2003) examined the prevalence of pain in depression, and vice versa, in an excellent and comprehensive review. This report examined over 42 studies reporting on the prevalence of major depression in chronic pain patients, demonstrating a prevalence which varied depending on the clinical setting and pain condition. For example, the mean prevalence of depression reported by chronic pain patients in pain clinics was 52%; in psychiatric clinics 38%; in population-based settings 18%; and in primary care 27%. Conversely, pain in depressed patients was considerably less studied (n=14 studies), reporting a range of 15 - 100%, with a mean of 65% (Bair et al., 2003). Since the publication of this comprehensive review, a number of in-depth studies have confirmed the high comorbidity of depression and pain (Table 1.1). In the general population, the prevalence of pain in depression ranges from 43 - 65% (Demyttenaere et al., 2006, Beesdo et al., 2010, Ohayon and Schatzberg, 2003), in primary care settings chronic pain is reported in 65 - 80% of depressed patients (Bair et al., 2004, Arnow et al., 2006, Gameroff and Olfson, 2006) and in clinical settings or in outpatient populations, the prevalence of pain in depressed patients is of the order of 60 - 80% and particularly prevalent in females (Aguera-Ortiz et al., 2011, Vaccarino et al., 2009, Leuchter et al., 2010, Husain et al., 2007). Thus, the prevalence of pain in depressed patients remains high across a variety of populations and
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treatment settings. With chronic pain, the prevalence of depression appears to increase as the setting moves from the community to tertiary care, suggesting that increasing pain severity requiring more specialised treatment may be associated with greater incidence of depression. In chronic pain patients, the prevalence of depression ranges from less than 3% up to 35% in the general population (Breivik et al., 2006a, McWilliams et al., 2003, Miller and Cano, 2009, Currie and Wang, 2004, Demyttenaere et al., 2007, Beesdo et al., 2010), almost 20% in primary care (Raftery et al., 2011, Rosemann et al., 2007), and increases to over 85% in patients being treated in specialised pain clinics (Radat et al., 2013, Poole et al., 2009, Ho et al., 2011). Fibromyalgia is a disorder of chronic, widespread pain and allodynia of yet unknown cause, with a female to male ratio of 9:1 (Yunus, 2002). This disorder is known to be consistently highly comorbid with depression, with up to 80% of patients suffering from depression (Aguglia et al., 2011, Fietta et al., 2007), making it a good disease model in which to study the clinical interaction between chronic pain and depression. Undiagnosed or medically unexplained pain can have particularly detrimental effects on patients, with over 80% of patients with undiagnosed pain also having a concomitant mood disorder, again a phenomenon more prevalent in females (Aguera et al., 2010, Aguera-Ortiz et al., 2013, Katon et al., 2001).
### Table 1.1 Prevalence of pain in depressed patients, and prevalence of depression in chronic pain patients. MDD – Major depressive disorder

<table>
<thead>
<tr>
<th>Patients</th>
<th>Population</th>
<th>Prevalence of pain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major depressive disorder (MDD) (literature review, n=14)</td>
<td>Primary care</td>
<td>15-100% (mean 65%)</td>
<td>(Bair et al., 2003)</td>
</tr>
<tr>
<td>MDD (n=5808)</td>
<td>Primary care</td>
<td>66%</td>
<td>(Arnow et al., 2006)</td>
</tr>
<tr>
<td>Depressive disorders (MDD and dysthymia, n=573)</td>
<td>Primary care</td>
<td>69%</td>
<td>(Bair et al., 2004)</td>
</tr>
<tr>
<td>MDD (n=207)</td>
<td>Primary care</td>
<td>69.1%</td>
<td>(Gumeroff and Olfson, 2006)</td>
</tr>
<tr>
<td>MDD (n=21,425)</td>
<td>General population (4% of which had MDD)</td>
<td>50%</td>
<td>(Demyttenaere et al., 2006)</td>
</tr>
<tr>
<td>MDD (n=508)</td>
<td>General population (Germany)</td>
<td>59.8% (lifetime, unexplained pain) 28.8% (diagnosed pain disorder)</td>
<td>(Beesdo et al., 2010)</td>
</tr>
<tr>
<td>MDD (n=18,980)</td>
<td>General population (Europe) (4% of which had MDD)</td>
<td>43%</td>
<td>(Ohayon and Schatzberg, 2003)</td>
</tr>
<tr>
<td>MDD (n=2469)</td>
<td>Community population</td>
<td>64.2%</td>
<td>(Chen et al., 2012b)</td>
</tr>
<tr>
<td>All types of DSM-IV-TR depressive disorders (n=3566)</td>
<td>Psychiatric outpatients;</td>
<td>59.1%</td>
<td>(Aguera-Ortiz et al., 2011)</td>
</tr>
<tr>
<td>MDD (n=2876)</td>
<td>Psychiatric outpatients</td>
<td>80%</td>
<td>(Leuchter et al., 2010)</td>
</tr>
<tr>
<td>MDD (n=3754)</td>
<td>Outpatients</td>
<td>77%</td>
<td>(Husain et al., 2007)</td>
</tr>
<tr>
<td>MDD (n=2191)</td>
<td>Patients in clinical trial for MDD treatment</td>
<td>57%</td>
<td>(Vaccarino et al., 2009)</td>
</tr>
<tr>
<td>Chronic pain (literature review, n=42)</td>
<td>Various settings</td>
<td>(Mean) 18% in general population 27% primary care 52% in pain clinics or inpatient pain programs 38% in psychiatric outpatient 56% in orthopaedic or rheumatology clinics 85% in dental clinics 13% in gynaecology clinics</td>
<td>(Bair et al., 2003)</td>
</tr>
<tr>
<td>Chronic pain (n=46,394)</td>
<td>General population</td>
<td>21%</td>
<td>(Breivik et al., 2006a)</td>
</tr>
<tr>
<td>Chronic pain (n=5877)</td>
<td>General population</td>
<td>20.2% (depression) 5.2% (dysthymia)</td>
<td>(McWilliams et al., 2003)</td>
</tr>
<tr>
<td>Chronic pain (n=1179)</td>
<td>General population</td>
<td>35%</td>
<td>(Miller and Cano, 2009)</td>
</tr>
<tr>
<td>Chronic pain (n=118,533)</td>
<td>General population</td>
<td>19.8%</td>
<td>(Currie and Wang, 2004)</td>
</tr>
<tr>
<td>Chronic neck/back pain (n=85,088)</td>
<td>General population</td>
<td>2.5% and 15.7%</td>
<td>(Demyttenaere et al., 2007)</td>
</tr>
<tr>
<td>Unexplained pain (n=1630)</td>
<td>General population</td>
<td>17.8% (12 month) 24.5% (12 month)</td>
<td>(Beesdo et al., 2010)</td>
</tr>
<tr>
<td>Chronic pain (n=1204)</td>
<td>Primary Care</td>
<td>15%</td>
<td>(Raftery et al., 2011)</td>
</tr>
<tr>
<td>Osteoarthritis (n=1021)</td>
<td>Primary care</td>
<td>19%</td>
<td>(Rosemann et al., 2007)</td>
</tr>
<tr>
<td>Neuropathic pain (n=182)</td>
<td>Multicentre cohort (patients)</td>
<td>47.2% (lifetime) 29.7% (current)</td>
<td>(Radat et al., 2013)</td>
</tr>
<tr>
<td>Chronic pain (n=36)</td>
<td>Pain clinic patients</td>
<td>72-86%</td>
<td>(Poole et al., 2009)</td>
</tr>
<tr>
<td>Chronic pain (n=107)</td>
<td>Pain clinic patients</td>
<td>62.9% (psychiatric disorder) 31.5% (depression)</td>
<td>(Ho et al., 2011)</td>
</tr>
<tr>
<td>Fibromyalgia (n=30)</td>
<td>Outpatients</td>
<td>83.3%</td>
<td>(Aguglia et al., 2011)</td>
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</table>
Depressed patients often present to primary care physicians with somatic complaints only – in fact, up to two thirds of patients report just somatic complaints, 60% of which are pain symptoms, with the psychological symptoms being ignored or denied (Simon et al., 1999, Bridges and Goldberg, 1985, Kirmayer et al., 1993, Hollifield et al., 1994). The presentation of only pain complaints results in a higher likelihood of an inaccurate diagnosis of a primary pain condition rather than depression (Tylee et al., 1995, Kirmayer et al., 1993, Bridges and Goldberg, 1985), leading to inappropriate medication or therapy and impaired treatment outcome (Kessler et al., 2003, Kroenke et al., 2008, Bair et al., 2004, Fava et al., 2004). In a longitudinal study, it was reported that 47% of patients with depression alone achieved recovery over time (treatments were not examined), compared to only 9% of patients exhibiting depression combined with painful symptoms (Geerlings et al., 2002). This is significant given that the presence of residual symptoms results in a greater likelihood of relapse (Keller, 2003), increased disability (Sobocki et al., 2006) and more suicide attempts (Kennedy and Foy, 2005). Patients with painful somatic illness are at a high risk of suicide (Stenager et al., 1994). Therefore, the presence of comorbid pain increases mortality and impedes remission in depression. Effective treatment of pain symptoms has been shown to result in higher remission rates in depressed patients (Russell et al., 2008, Fava et al., 2004). Comorbid patients are more likely to use healthcare services and have increased absenteeism (Aguera et al., 2010, Demyttenaere et al., 2006, Bao et al., 2003); report multiple pain complaints and increased frequency and duration of pain which further increases the probability of depression (Dworkin and Gitlin, 1991, Kroenke and Price, 1993, Vaccarino et al., 2009); and experience higher pain severity which is associated with more severe depression, more pain-related functional limitations, and poorer health (Von Korff et al., 1992, Vaccarino et al., 2009). The comorbidity of depression and pain increases the socioeconomic cost than either disorder alone. For example, the presence of depression in chronic pain patients leads to double the costs (direct and indirect) compared to those without depression (€9,214 vs. €4,176 per annum).
(Raftery et al., 2012), and direct medical care costs of patients with major depressive disorder (MDD) and moderate pain-related interference were over twice as high as those for patients with MDD and no pain (Gameroff and Olfson, 2006).

Despite the high incidence of comorbidity of these two illnesses, the physiological link and the substrates mediating the interaction between pain and depression remain ambiguous. As discussed, the combination of depression and pain is more disabling and costlier to both patients and society than either alone, highlighting the need for further knowledge of the underlying mechanisms facilitating depression and chronic pain comorbidity, in order to ensure the correct therapeutic response and development of novel treatments. Three main theories attempt to explain the relationship between depression and pain: (1) that depression is caused by chronic pain, a stressor; (2) that pain arises as a somatic manifestation of depression; and (3) depression and pain are independent processes which share pathophysiological mechanisms (Bair et al., 2003) (Fig. 1.2). Indeed, as pain and depression frequently co-occur, exacerbate one another and respond to the same medications, this further supports common neural substrates. Shared pathways provide a means through which pain can influence mood and lead to depression/anxiety, and by the same process allows mood to significantly impact on the pain experience. With mood disorders and chronic pain, pathological changes in the central nervous system may influence normal processing of mood and/or pain, leading to an increased vulnerability to either condition. In this chapter, clinical studies reporting alterations in experimental pain responding in depressed patients will be examined and evidence for altered nociceptive responding in animal models of depression will be provided. The neural substrates that may be responsible for the pain-depression dyad will be discussed, including common neuroanatomical structures, neurotransmitters, inflammation and other shared systems implicated in emotion and nociception.
Fig. 1.2. Proposed relationship between depression and chronic pain.
1.1.1 Altered pain responding in depression

Disruption of pain processing has been investigated in patients with primary depression by examining thresholds and tolerance of noxious and innocuous stimuli (Table 1.2). Pain threshold relates to the least experience of pain that can be identified by a subject, while pain tolerance is defined as the highest level of pain that a subject is prepared to tolerate (International Association for the Study of Pain). The majority of studies have examined experimental pain thresholds or pain tolerance of depressed patients to mechanical, heat and cold stimuli applied to the skin, with some studies determining responses to deep ischaemic or muscle pain. The effect of depression on sensory processing, pain perception and tolerance is a controversial topic. In the literature, the response of depressed patients to experimental pain is inconsistent, with reports of increases, decreases and no change in thresholds and tolerance. This is in stark contrast to the strong association of depression with increased clinical and chronic pain complaints.

While many studies report enhanced pain sensitivity (i.e. reduced thresholds and tolerance) to mechanical and thermal stimuli applied to the skin (Table 1.2), in support of the theory that depressed patients are more sensitive to pain, a number of studies reported the converse. That is, a paradoxical increase in heat, cold and pressure pain thresholds of the skin, suggesting that depressed patients are less sensitive to painful stimuli applied to the skin (Table 1.2), despite these experimental groups reporting concomitant clinical or physical pain complaints (Lautenbacher et al., 1999, Abbott and Lehoux, 2011, Kundermann et al., 2009, Dickens et al., 2003). These alterations in pain thresholds are not due to changes in sensory detection thresholds (e.g. non-painful warmth), but specific to nociception (e.g. painful heat) (Table 1.2). However, it has also been reported that although absolute sensory thresholds were increased, relative pain perception thresholds (expressed as a ratio of sensory : pain perception thresholds) were significantly reduced in depressed patients (Adler and Gattaz, 1993), suggesting a more complex response to experimental pain in depression. In
addition, no change in pressure or thermal pain thresholds has been reported (Giesecke et al., 2005, Strigo et al., 2008a). As depression is a heterogeneous and complex disorder, pain responding may differ between subgroups of depressed patients. Thus, the induction of sad mood (using sad music, words, images etc.) in nondepressed subjects has been used to identify how emotion alters pain thresholds or perception. The induction of a depressed mood reliably causes reduced thresholds and enhances the perceived unpleasantness of noxious thermal (heat and cold) stimuli applied to the skin in nondepressed subjects (Wagner et al., 2009, Willoughby et al., 2002, Pinerua-Shuhaibar et al., 2011). In addition, the induction of a depressed mood resulted in lower pain tolerance and increased pain perception in depressed (Terhaar et al., 2010) and chronic pain patients (Kenntner-Mabiala et al., 2008, Tang et al., 2008). Despite the aforementioned discrepancies for nociceptive threshold of stimuli applied to the skin, there appears to be greater consensus for increased sensitivity to deep somatic pain, such as ischaemic or deep muscle pain in depressed patients (Pinerua-Shuhaibar et al., 1999, Bar et al., 2005, Bar et al., 2011, Normand et al., 2010, Janke et al., 2004, Gormsen et al., 2004, Suarez-Roca et al., 2003). Indeed, Bar and colleagues found that pain perception in major depressive disorder depends on modality, reporting hypoalgesia to heat and electrical pain but hyperalgesia to ischaemic muscle pain in depressed patients (Bar et al., 2005).

As highlighted earlier, pain involves both sensory and emotional components and although pain thresholds may be unaltered or even increased in some depressed patients, it is important to note that the emotional or affective response to experimental pain may be significantly transformed in the presence of a mood disorder. Only a handful of studies have investigated both the emotional and somatic characteristics of pain in depression. Increased negative affect to a normally innocuous heat stimulus (Strigo et al., 2008b) and enhanced unpleasantness to a noxious thermal stimulus (Pinerua-Shuhaibar et al., 2011) is reported in depressed individuals. Moreover, increased negative affect was associated with lower
heat pain thresholds (Strigo et al., 2008b). Although pain responding in the cold pressor test was unaltered in subjects with low mood, these individuals reported increased distress (Abbott and Lehoux, 2011), and despite unchanged ischemic pain thresholds or intensity ratings, tolerance and subjective pain ratings were increased in subjects with minor depression (Pinerua-Shuhaibar et al., 1999). Of particular interest are studies reporting that despite a reduced sensitivity to painful cold (Boettger et al., 2013) and heat (Bar et al., 2011) stimuli, depressed patients had a tendency to report higher subjective ratings of pain and increased unpleasantness of pain (Boettger et al., 2013, Bar et al., 2011). Therefore, although depressed patients exhibit decreased, increased and no change in pain thresholds of the skin, a consensus exists thus far that the emotional and unpleasant quality of pain is enhanced in depression. These studies emphasise the need to assess the emotional/affective component of pain when measuring thresholds to experimental pain, particularly in the context of mood disorders.

Gender is an important and often overlooked factor in clinical studies examining experimental pain responding. Females are twice as likely to suffer depression compared to males (Munce and Stewart, 2007), have greater pain sensitivity (Fillingim et al., 2009), and are more likely to experience chronic pain (Tsang et al., 2008, Munce and Stewart, 2007). The need to examine the association between depression, pain and gender has been noted in the literature (Fillingim et al., 2009) and the current evidence is scant. Female gender bias is observed with increased heat pain thresholds in depressed patients detected mainly in females (Bar et al., 2003) and females with fibromyalgia displaying reduced pressure pain thresholds and higher levels of depressive symptoms than males, although these were not correlated with one another (Castro-Sanchez et al., 2012). Although depression and female gender both result in lower pressure pain thresholds, no interaction effect was found (Euteneuer et al., 2010, Hennings et al., 2012). In addition, depressive symptoms in a non-clinical population were not found to alter pain thresholds or tolerance in either gender (Thibodeau et al., 2013), and no gender differences were found in the ischaemic pain threshold, tolerance, intensity, or unpleasantness reported by depressed patients (Pinerua-Shuhaibar et al., 1999). Thus, the current literature is
conflicted, and further research is required to clarify if sex or gender mediates the relationship between depression and pain.

Only a handful of studies have examined the effect of antidepressant treatment on pain thresholds in depressed patients, and as yet no studies have been placebo controlled. Depressed patients exhibited increased heat and decreased ischemic pain thresholds, effects normalised and unchanged by chronic duloxetine (selective serotonin and noradrenaline reuptake inhibitor, SSNRI) treatment, respectively (Bar et al., 2011). Pressure pain thresholds are reported to normalise following different types of antidepressant treatment (selective serotonin reuptake inhibitors [SSRI], noradrenaline reuptake inhibitors [NRI], electroconvulsive therapy [ECT]) in depressed patients when compared to baseline (Graff-Guerrero et al., 2008, Schreiber et al., 2003). However, one study reported no change in pressure or cold pain thresholds following ECT compared to baseline despite significant improvement in symptoms of depression (Gormsen et al., 2004). Thus, the effect of treatment on nociceptive thresholds in depression is inconclusive and more studies are required, particularly in patients with clinical pain complaints, to elucidate the effect of successful antidepressant treatment on altered nociceptive responding in depression. Antidepressants are used in the treatment of chronic pain conditions in both depressed and nondepressed individuals (for review see Briley and Moret, 2008, Mico et al., 2006). Although less well studied, antidepressants have also been shown to exhibit analgesic effects in experimental pain tests in nondepressed subjects. For example, in healthy subjects, acute treatment with a selective noradrenaline reuptake inhibitor (SNRI, venlafaxine) or tricyclic antidepressant (TCA, imipramine) increased electrical, pressure, heat and/or cold pain thresholds (Enggaard et al., 2001a, Poulsen et al., 1995, Bromm et al., 1986, Enggaard et al., 2001b), and chronic amitriptyline treatment (TCA) attenuated electrical pain thresholds in healthy volunteers (Gorelick et al., 1998). In patients with clinical pain conditions, chronic duloxetine (a selective serotonin and norepinephrine reuptake inhibitor, SSNRI) reduces pressure pain thresholds in fibromyalgia patients in one study (Arnold et al., 2004) but not another (Russell et al., 2008). Chronic antidepressant
treatment (venlafaxine, duloxetine, imipramine) in neuropathic and phantom pain patients decreases pressure and pin-prick hyperalgesia, heat, cold and/or electrical thresholds (Wilder-Smith et al., 2005, Yucel et al., 2005, Sindrup et al., 2003, Vranken et al., 2011), and chronic amitriptyline treatment reduces experimental muscle pressure pain in chronic tension headache patients (Gobel et al., 1994). As such, treatment with antidepressants has been shown to alter experimental pain responding when administered acutely or chronically in healthy and chronic pain populations, thus it would be of interest to further examine the effect of treatment on pain responding in depressed patients.

Although the examination of experimental pain responding can be useful to detect alterations in sensory and nociceptive processing, this measure of pain differs from the experience of clinically reported pain experienced by depressed patients in a number of ways. Experimental pain is predictable, subjects are notified that no tissue damage will occur and have control over stopping the stimulation, and acute experimental pain does not have the emotional, cognitive, social and behavioural factors that accompany a chronic pain condition. However, a greater nociceptive flexion reflex in fibromyalgia patients correlated with a higher level of pain intensity and presence of depressive symptoms (Ang et al., 2011), suggesting that pathological alterations in nociceptive responding are related to central processing of pain and depression. Pathological changes in the central nervous system in depression that are also involved in pain processing may lead to persistent pain in depressed patients. Supporting this hypothesis, Klauenberg and colleagues reported increased wind-up (an increase in pain intensity when a painful stimulus is delivered repeatedly) following repeated noxious mechanical stimulation in patients with depression, despite an unchanged mechanical pain threshold (Klauenberg et al., 2008). Furthermore, depressed patients showed significantly decreased Aδ-laser evoked amplitudes and increased C-fibre laser evoked latencies, suggesting that Aδ alterations may represent increases in superficial pain thresholds of the skin during depression, whereas the C-fibre component mediates the
deep somatic pain processing (Terhaar et al., 2011). Ischaemic pain resulted in a blunted cardiovascular autonomic response in depressed patients compared to healthy controls (Pinerua-Shuhaibar et al., 1999), whereas electric stimuli resulted in a stronger sympathetic response of the skin of depressed patients compared to controls, despite reduced pain perception in these patients (Boettger et al., 2010), effects which may further explain the differential pain responding to superficial (electrical) vs. deep somatic (ischaemic) pain. Diffuse noxious inhibitory controls are an endogenous pain control mechanism, occurring when a painful stimulus is inhibited when another nociceptive stimulus is applied to a distant part of the body (Le Bars et al., 1981, Villanueva and Le Bars, 1995), and may be mediated by descending monoaminergic projections (Chitour et al., 1982). Diffuse noxious inhibitory controls are reportedly not altered in depression, although deficits are observed in fibromyalgia (Normand et al., 2010). Deficits in pain inhibition in fibromyalgia patients were more pronounced in patients with comorbid depressive symptoms (de Souza et al., 2009). In contrast to Normand et al., a deficit of descending inhibition (measured by the jaw opening reflex) was observed in acutely depressed unmedicated patients (Bar et al., 2003). Alterations in activity (measured by imaging cerebral blood flow) in brain regions that modulate emotional responding may also impact on nociceptive pathways and modulate responding, as seen with heightened activation of certain brain areas in anticipation of pain in depressed patients (Strigo et al., 2008a), which will be discussed in greater detail below, along with other common substrates implicated in depression and pain.
<table>
<thead>
<tr>
<th>Patients</th>
<th>Test</th>
<th>Result</th>
<th>Notes</th>
<th>Ref</th>
</tr>
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<tbody>
<tr>
<td><strong>REDUCED SENSITIVITY</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Major depression</td>
<td>Contact noxious heat</td>
<td>↑ Pain thresholds</td>
<td></td>
<td>(Lautenbacher et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Non-noxious warm/cold/vibration</td>
<td>⇨ Sensory thresholds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major depression</td>
<td>Heat/cold pain thresholds</td>
<td>↑ Heat, but not cold, pain thresholds</td>
<td>No effect of current pain complaints or mood</td>
<td>(Kundermann et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Warmth/cold detection thresholds</td>
<td>⇨ Sensory thresholds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDD</td>
<td>Pressure/cold/heat pain thresholds</td>
<td>↑ Pain thresholds to all modalities</td>
<td>↑ frequency, intensity and unpleasantness of clinical pain complaints</td>
<td>(Lautenbacher et al., 1999)</td>
</tr>
<tr>
<td>MDD</td>
<td>Heat/cold pain thresholds</td>
<td>↑ Heat and cold pain thresholds</td>
<td>Tendency for greater subjective pain rating</td>
<td>(Boettger et al., 2013)</td>
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<tr>
<td></td>
<td>Thermal grill illusion</td>
<td>⇨ Perceived as less painful</td>
<td>Tendency for lower subjective pain rating</td>
<td></td>
</tr>
<tr>
<td>MDD</td>
<td>Cold pain</td>
<td>↑ Pain thresholds</td>
<td></td>
<td>(Schwier et al., 2010)</td>
</tr>
<tr>
<td>MDD</td>
<td>Heat pain thresholds</td>
<td>↑ Pain thresholds</td>
<td></td>
<td>(Terhaar et al., 2010, Bar et al., 2007, Bar et al., 2005, Bar et al., 2003, Bar et al., 2011)</td>
</tr>
<tr>
<td>MDD</td>
<td>Electrical pain threshold and tolerance</td>
<td>↑ Pain threshold and tolerance</td>
<td></td>
<td>(Bar et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Marazziti et al., 1998, Boettger et al., 2010)</td>
</tr>
<tr>
<td>Low mood</td>
<td>Punctuate tactile thresholds</td>
<td>↑ Tactile perception thresholds</td>
<td>↑ Clinical pain complaints</td>
<td>(Abbott and Lehoux, 2011)</td>
</tr>
<tr>
<td></td>
<td>Pressure/cold pain thresholds</td>
<td>⇨ Pain thresholds</td>
<td>↑ Distress in cold pressor test</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neurogenic inflammation induced by capsaicin</td>
<td>⇨ Response to topical capsaicin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDD</td>
<td>Laser heat stimuli</td>
<td>↑ Thresholds</td>
<td></td>
<td>(Terhaar et al., 2011, Weiss et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>⇨ Perception</td>
<td></td>
<td></td>
</tr>
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</table>
### Table 1.2 Experimental pain responding in depressed patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Test</th>
<th>Result</th>
<th>Notes</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INCREASED SENSITIVITY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDD</td>
<td>Pressure/cold/heat pain thresholds</td>
<td>↓ Pressure pain thresholds</td>
<td></td>
<td>(Spernal et al., 2003)</td>
</tr>
<tr>
<td>MDD</td>
<td>Heat sensory/pain thresholds</td>
<td>↓ Pain thresholds, ↑ Unpleasantness/intensity, ↑ Sensory thresholds</td>
<td></td>
<td>(Strigo et al., 2008b)</td>
</tr>
<tr>
<td>MDD</td>
<td>Heat pain</td>
<td>↓ Tolerance, ↓ Thresholds</td>
<td></td>
<td>(Borckardt et al., 2005, Paul-Savoie et al., 2011)</td>
</tr>
<tr>
<td>Depression</td>
<td>Pressure pain thresholds</td>
<td>↓ Thresholds</td>
<td></td>
<td>(Chiu et al., 2005, Hennings et al., 2012, Euteneuer et al., 2010)</td>
</tr>
<tr>
<td>Severe depression</td>
<td>Pressure pain detection and tolerance, and cold pressor test</td>
<td>↓ Pressure and cold pain tolerance, ↓ pressure detection thresholds</td>
<td></td>
<td>(Gormsen et al., 2004)</td>
</tr>
<tr>
<td>MDD</td>
<td>Thermal pain thresholds, cold pressor test</td>
<td>↓ pain thresholds</td>
<td></td>
<td>(Normand et al., 2010)</td>
</tr>
<tr>
<td>Minor depression</td>
<td>thermal grill illusion (non-noxious unpleasant burning sensation)</td>
<td>↑ Pain perception, ↑ unpleasantness</td>
<td></td>
<td>(Pinerua-Shuhaibar et al., 2011)</td>
</tr>
<tr>
<td>Depression</td>
<td>Muscle tenderness and pressure pain thresholds</td>
<td>↑ tenderness, ↓ unpleasantness</td>
<td>Blunted autonomic response to pain, ↑ Clinical pain complaints</td>
<td>(Pinerua-Shuhaibar et al., 1999)</td>
</tr>
<tr>
<td>Minor depression</td>
<td>Ischaemic pain</td>
<td>⇓ Pain threshold, intensity or unpleasantness, ↓ Tolerance, ↑ Pain ratings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDD</td>
<td>Ischaemic pain</td>
<td>↓ Threshold and tolerance</td>
<td></td>
<td>(Bar et al., 2005, Bar et al., 2011)</td>
</tr>
<tr>
<td>Minor depression</td>
<td>Intensity of thermal and tactile post-ischaemic paraesthesias</td>
<td>↑ Thermal (warm/cool) and tactile (tingling) paraesthesias</td>
<td></td>
<td>(Suarez-Roca et al., 2003)</td>
</tr>
<tr>
<td><strong>NO CHANGE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibromyalgia patients with depression</td>
<td>Pressure pain</td>
<td>⇔</td>
<td></td>
<td>(Giesecke et al., 2005)</td>
</tr>
<tr>
<td>MDD</td>
<td>Thermal pain</td>
<td>⇔ ↑ unpleasantness to non-noxious warmth</td>
<td></td>
<td>(Strigo et al., 2008a)</td>
</tr>
</tbody>
</table>
1.2 Preclinical studies support clinical data on the association between depression and pain and vice versa

Evidence from animal models supports clinical data on the relationship between depression and pain and provides a means to study the underlying mechanisms and neural substrates mediating the association between these disorders. A variety of animal models of chronic pain have been demonstrated to display depressive- and anxiety-like behaviour, while conversely, several animal models of depression have been shown to exhibit altered nociceptive responding, lending further credence to a biological basis underpinning depression-pain comorbidity.

1.2.1 Affective behavioural changes in models of chronic pain

Animal models of persistent chronic pain include inflammatory pain (formalin test, complete Freund’s adjuvant, carrageenan) and neuropathic pain (which arises from damage to peripheral nerves: chronic constriction injury, sciatic nerve cuffing, spinal nerve ligation/transection, spared nerve injury, partial nerve injury). Presented in Table 1.3 is an overview of the studies in which affective/emotional behavioural responding has been assessed in models of chronic pain. Affective behavioural changes appear to be time dependent, as anxiety- and depressive-like behaviours manifest several months after the induction of neuropathy, whereas mechanical and thermal hypersensitivity occurs earlier.

Several models of neuropathic pain have been shown to exhibit depressive-like behaviour exemplified as increased immobility in the forced swim test and reduced sucrose preference, a measure of anhedonia (see Table 1.3), with a few exceptions as measured in the saccharine preference test (Gregoire et al., 2012) and tail-suspension test (Benbouzid et al., 2008, Hasnie et al., 2007b), which are similar tests to the aforementioned assays. In addition, depressive-like behaviour in the forced swim test was not altered when examined 2 weeks post-surgery (Kontinen et al., 1999). In contrast, in a model of persistent inflammatory pain (complete Freund's adjuvant), depressive-like behaviour is observed at 1 and 2 weeks post-
treatment (Kim et al., 2012). Moreover, chronic stress has been shown to exacerbate pain-related depressive-like behaviour in this model (Kim et al., 2012), an effect also seen in a model of neuropathic pain (Norman et al., 2010b). Recent data has reported that the increased immobility in the chronic constriction injury (CCI) model is at the expense of climbing, but not swimming, behaviour (Hu et al., 2009, Alba-Delgado et al., 2013), suggesting that this effect may be mediated by a deficit in noradrenergic neurotransmission in this model (Detke et al., 1995). In addition, depressive-like behaviour has been reported in mice following sciatic nerve cuffing as exemplified by reduced grooming behaviour in the splash test and an increased latency to feed in an aversive environment (Yalcin et al., 2011). Thus, depressive-like behaviour is observed in models of persistent pain, in a time-dependent manner.

A wealth of data has demonstrated anxiety-like behaviour in models of neuropathic and inflammatory pain in a variety of tests such as the elevated plus maze, open field, light-dark box, marble-burying test and social interaction test (Table 1.3). These tests are based on the aversion of the rodent to bright, open spaces, and decreased time in these aversive spaces (open arms of the elevated plus/zero maze, inner zone of the open field, light chamber of the light-dark box) indicates increased anxiety-like behaviour. Increased marble burying behaviour and decreased social interaction are also putative behavioural indices of anxiety in rodents. It should be noted that some studies report no change in anxiety-related behaviour, an effect that may be due to the time-point, age of the animal, model or test in which these behaviours are examined. For example, in the spared nerve injury model, young and old, but not mid-aged, rats exhibit reduced open arm entries in the elevated plus maze at 5 weeks post-injury. In addition, anxiety-like behaviour in the open field and elevated plus maze is unaltered from 1 - 7 weeks post-injury (Norman et al., 2010b, Goncalves et al., 2008, Norman et al., 2010a, Leite-Almeida et al., 2009), however at 9 weeks anxiety-like behaviour is observed, an effect coincident with decreased volume of the frontal cortex (Seminowicz et al., 2009). Across various models of persistent pain, 4 weeks appears to be the most common
time-point at which anxiety-like behaviours are observed (Table 1.3). At this time-point, anxiety-like behaviours are exemplified by reduced time in the open arms of the elevated plus maze in the chronic constriction injury (CCI), spared nerve injury (SNI), spinal nerve ligation (SNL), partial sciatic nerve ligation (pSNL) models of neuropathic pain and complete Freund’s adjuvant (CFA) model of inflammatory pain. Indeed, time-dependent manifestations of both anxiety- and depressive-like behaviour are reported (Yalcin et al., 2011, Suzuki et al., 2007, Narita et al., 2006b). This delay suggests that persistent pain induces alterations in supraspinal structures and pathways which modulate emotion. Moreover, the depressive-like phenotype and anxiogenic behaviour in these animals is reversed by antidepressants (amitriptyline, duloxetine, fluoxetine), morphine, gabapentin, diazepam and ketamine (an N-methyl-D-aspartate receptor antagonist) (Wang et al., 2011, Hu et al., 2010, Jesse et al., Shi et al., 2010b, Gregoire et al., 2012, Ceci et al., 2008, Rice et al., 2008), further supporting shared pathways, which will be discussed in greater detail below.

The affective or emotional component of pain can be modelled in rodents by examining avoidance of a chamber associated with noxious stimulation in the place escape/avoidance paradigm which can be observed at the same time of onset as allodynia and hyperalgesia (LaBuda and Fuchs, 2000a), and conditioned place aversion to a pain-paired environment with increased number of hind-paw withdrawals and fewer novel object interactions during painful conditioning sessions, which is maintained for up to 1 month (Hummel et al., 2008). Affective pain behaviour has been reported in neuropathic and chronic inflammatory pain models, exemplified as increased avoidance in the place escape/avoidance and increased negative affective behaviour in the conditioned place aversion paradigm (Alba-Delgado et al., 2013, Pedersen and Blackburn-Munro, 2006, LaBuda and Fuchs, 2005, Hummel et al., 2008, Parent et al., 2012, Narita et al., 2006a). This behaviour has been measured from 2 days until 3 weeks following injury. However, it has been reported that at the time of onset of anxiety- and depressive-like behaviour (4 weeks), increased avoidance of the noxious painful stimulus is switched to avoidance of the aversive light chamber (Alba-Delgado et al., 2013), indicating a complex interaction
between emotion and pain.

Affective pain behaviour can be attenuated by analgesics (morphine), anti-inflammatory (diclofenac, celecoxib), anticonvulsants (gabapentin) and antidepressants (duloxetine) (Pedersen and Blackburn-Munro, 2006, Boyce-Rustay et al., 2009, Hummel et al., 2008), thus these pharmacological treatments work on both the somatic and affective components of pain. Further evidence suggests that the affective component of chronic pain can be attenuated independently of the sensory component. Examination of dose-response relationships indicates that higher doses of analgesic/antidepressant/anti-inflammatory agents (celecoxib, diclofenac, duloxetine, fluoxetine and morphine) are required to attenuate mechanical allodynia associated with neuropathic pain than that required to ameliorate affective pain behaviour (Boyce-Rustay et al., 2009, Hummel et al., 2008), suggesting that the affective and sensory components of pain are distinct, but parallel, pathways.
# Models of chronic pain

<table>
<thead>
<tr>
<th>Models of chronic pain</th>
<th>Depression/ anxiety-like behaviour</th>
<th>Test</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chronic constriction injury</strong></td>
<td>Depressive-like behaviour</td>
<td>FST</td>
<td>↑ immobility (2 and 3 weeks post-injury)</td>
<td>(Jesse et al., 2010, Fukuhara et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Depression-like behaviour</td>
<td>FST</td>
<td>↑ immobility at the expense of climbing behaviour (3-4 weeks)</td>
<td>(Hu et al., 2009, Alba-Delgado et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>Saccharin preference test</td>
<td>No change (2-3 weeks)</td>
<td>(Gregoire et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>EPM</td>
<td>↓ time in open arms (3-4 weeks)</td>
<td>(Ceci et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>Elevated zero maze</td>
<td>↓ time in open arms (4 weeks)</td>
<td>(Alba-Delgado et al., 2013, Gregoire et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>OF</td>
<td>↑ time and entries to inner zone (2-3 weeks)</td>
<td>(Gregoire et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Affective pain behaviour</td>
<td>PEAP</td>
<td>↑ time in light (1-3 weeks), ↓ time in light (4 weeks)</td>
<td>(Alba-Delgado et al., 2013, Pedersen and Blackburn-Munro, 2006)</td>
</tr>
<tr>
<td><strong>Sciatic nerve cuffing</strong></td>
<td>Depressive-like behaviour</td>
<td>FST</td>
<td>↑ immobility (8 and 9 weeks post-injury)</td>
<td>(Yalcin et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>TST</td>
<td>No change in immobility at 3 or 5 weeks</td>
<td>(Benbouzid et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>Splash test</td>
<td>↓ grooming (6 and 8 weeks)</td>
<td>(Yalcin et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>EPM</td>
<td>↓ time in open arms (4 weeks)</td>
<td>(Benbouzid et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Novelty-supressed feeding</td>
<td></td>
<td>↑ latency to feed (5 and 8 weeks)</td>
<td>(Yalcin et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Light-dark box</td>
<td></td>
<td>↓ time in light (at 4, 7 and 8 weeks)</td>
<td>(Yalcin et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Marble-burying test</td>
<td></td>
<td>↑ burying (at 4 and 6 weeks)</td>
<td>(Benbouzid et al., 2008)</td>
</tr>
<tr>
<td><strong>Spared nerve injury</strong></td>
<td>Depressive-like behaviour</td>
<td>FST</td>
<td>↑ immobility (1, 2, 5 and 7 weeks)</td>
<td>(Norman et al., 2010a, Leite-Almeida et al., 2009, Goncalves et al., 2008, Wang et al., 2011, Norman et al., 2010b)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>Sucrose preference</td>
<td>↓ sucrose preference (2 and 7 weeks)</td>
<td>(Wang et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>EPM</td>
<td>↓ time in open arms (5 weeks), ↓ open arm entries (9 weeks)</td>
<td>(Leite-Almeida et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>OF</td>
<td>No change (7 weeks), No change (1, 5 or 7 weeks)</td>
<td>(Seminowicz et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td></td>
<td>↓ time inner (9 weeks)</td>
<td>(Goncalves et al., 2008)</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Models of chronic pain</th>
<th>Depression/ anxiety-like behaviour</th>
<th>Test</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinal nerve ligation /transection</td>
<td>Degenerative-like behaviour</td>
<td>FST</td>
<td>↑ immobility (2, 4 and 8 weeks)</td>
<td>(Hu et al., 2010, Suzuki et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>EPM</td>
<td>No change (2 weeks)</td>
<td>(Kontinen et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OF</td>
<td>↓ time and no. entries to open arms (4 and 8 weeks)</td>
<td>(Suzuki et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light-dark box</td>
<td>↓ time in inner zone (2, 4 and 8 weeks)</td>
<td>(Suzuki et al., 2007, Hasnie et al., 2007a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No change (2 weeks)</td>
<td>(Suzuki et al., 2007, Kontinen et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ time and no. entries to light (4 and 8 weeks)</td>
<td>(Suzuki et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No change (2 weeks)</td>
<td>(Kontinen et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PEAP</td>
<td>↑ time in light (day 3 and 4)</td>
<td>(Labuda and Fuchs, 2005, Labuda and Fuchs, 2000a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Conditioned place aversion</td>
<td>↑ avoidance of pain-related chamber (3 weeks)</td>
<td>(Hummel et al., 2008)</td>
</tr>
<tr>
<td>Partial sciatic nerve ligation</td>
<td>Degenerative-like behaviour</td>
<td>TST</td>
<td>No change (1, 2, 4 weeks)</td>
<td>(Hasnie et al., 2007b)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>EPM</td>
<td>↓ time open arms (4 weeks)</td>
<td>(Narita et al., 2006b, Matsuzawa-Yanagida et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light-dark box</td>
<td>↓ time in light (4 weeks)</td>
<td>(Matsuzawa-Yanagida et al., 2008, Narita et al., 2006b, Hasnie et al., 2007b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OF</td>
<td>No change (1, 2, 4 weeks)</td>
<td>(Hasnie et al., 2007b)</td>
</tr>
<tr>
<td>Complete Freund's adjuvant</td>
<td>Degenerative-like behaviour</td>
<td>FST</td>
<td>↑ immobility (1 and 2 weeks)</td>
<td>(Kim et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sucrose preference</td>
<td>Exacerbated chronic-stress induced anhedonia (1 week)</td>
<td>(Kim et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Anxiety-like behaviour</td>
<td>EPM</td>
<td>↓ time OA (4 weeks)</td>
<td>(Parent et al., 2012, Narita et al., 2006a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light-dark box</td>
<td>↓ time or no change in light chamber (4 weeks)</td>
<td>(Parent et al., 2012, Narita et al., 2006a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OF</td>
<td>↓ entries to inner zone (4 weeks)</td>
<td>(Parent et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Social interaction</td>
<td>↓ social interaction (4 weeks)</td>
<td>(Parent et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Affective pain behaviour</td>
<td>PEAP</td>
<td>↑ avoidance (day 2 and 3)</td>
<td>(Chen et al., 2012a, Boyce-Rustay et al., 2009)</td>
</tr>
</tbody>
</table>

Table 1.3 Affective behavioural changes in animal models of persistent pain. EPM Elevated plus maze; FST Forced Swim Test; OF Open Field; PEAP Place escape/avoidance arena; TST Tail suspension test
1.3 Altered nociceptive responding in models of depression
An overview of nociceptive responding in animal models of depression is presented in Table 1.4. Here, the main findings reported in the literature will be discussed.

1.3.1 Stress-related depressive-like phenotypes
Stress is a well-known predisposing factor to depression, and thus various stressful regimes have been employed in order to precipitate depressive-like behaviour in rodents. Chronic mild stress (CMS) is a procedure where rodents are subjected to a battery of unpredictable and mild stressors such as wet bedding, food and water deprivation, disruption of the dark-light cycle and/or having an intruder rat placed in the home cage (Willner et al., 1987). Chronic (6 week) exposure to this regimen induces depressive-like behaviour, the most consistently shown is a decrease in the consumption of sweet solutions, hypothesised to reflect anhedonia (Willner, 1997). CMS has also been shown to result in reduced body weight, decreased intracranial self-stimulation suggesting reward deficits, and decreased locomotor activity (Willner, 1997). In addition, chronic restraint stress (which is stress of a predictable nature) results in anxiety- and depressive-like behaviours such as decreased entries to the open arms of the EPM and increased immobility in the forced swim test (Chiba et al., 2012).

Rodents exposed to chronic unpredictable/predictable stress exhibit increased responding to an innocuous cold stimulus in the acetone drop test indicating cold allodynia (Bardin et al., 2009, Bravo et al., 2012) and reduced withdrawal thresholds to mechanical stimulation in the von Frey and paw pressure tests indicating mechanical allodynia and hyperalgesia, respectively (Kim et al., 2013, Spezia Adachi et al., 2012, Bardin et al., 2009, Imbe et al., 2012) (Table 1.4). In contrast, CMS results in decreased sensitivity to a noxious heat stimulus (thermal hypoalgesia), exemplified as an increased latency to respond in the hot plate (Shi et al., 2010b) and Hargreaves test (Qi et al., 2013), prior to and following intraplantar administration of complete Freund's adjuvant (Shi et al., 2010b). Similarly, unpredictable stress in the form of forced swim, overcrowding or restraint has been shown to increase the latency to respond to a noxious thermal
stimulus applied to the tail in the tail-flick test (Pinto-Ribeiro et al., 2004). CMS in mice selectively bred for high swim-stress induced analgesia results in lower hot plate latencies with concurrent depressive-like behaviour in the tail suspension test (Sacharczuk et al., 2009). Chronic restraint stress has been shown to reliably result in increased sensitivity to thermal stimuli as reduced thresholds are reported in the hot plate, tail flick and tail immersion tests (Spezia Adachi et al., 2012, Gamaro et al., 1998, Imbe et al., 2004, Costa et al., 2005, da Silva Torres et al., 2003, Bardin et al., 2009). Intraplantar formalin consistently results in enhanced spontaneous nociceptive behaviour in rats exposed to CMS (Shi et al., 2010b, Qi et al., 2013) and chronic restraint stress (Gameiro et al., 2006, Bardin et al., 2009, Gameiro et al., 2005). Moreover, electrical stimulation of the dura mater, a model of trigeminovascular nociception, results in enhanced nociceptive behaviour (head flicking and grooming) in CMS rats (Zhang et al., 2013). However, peripheral nerve injury-induced allodynia/hyperalgesia to mechanical and thermal stimuli is attenuated (Shi et al., 2010b) and unaltered (Bravo et al., 2012) following UCMS. Differences in the experimental design or the model of neuropathic pain (SNL vs. CCI) may account for this discrepancy. Shi et al carried out SNL surgery on rats first and then one week later initiated the stress protocol, with the increase in thresholds observed from week 3. In contrast, in the study by Bravo et al, stress and CCI were applied at the same time and monitored for 2 weeks. Despite no effect of CMS on CCI-induced mechanical allodynia, avoidance behaviour to a noxious mechanical stimulus is increased following chronic stress, suggesting enhanced affective pain behaviour in a model of depression (Bravo et al., 2012). The differences in pain responding observed in these studies may be due to stress-induced analgesia, believed to be an adaptive protective mechanism mediated by the descending pain pathways (Butler and Finn, 2009, Amit and Galina, 1986) or stress-induced hyperalgesia, a maladaptive response resulting in enhanced pain behaviour (Imbe et al., 2006). Differences in pain responding in models of chronic stress may relate to the predictability, type, intensity and duration of the stressor which exerts a complex influence on nociceptive, inflammatory and
neuropathic nociceptive responding depending on the stimulus modality, model and test employed.

Psychosocial stress plays an important role in many cases of clinical depression (Coyne and Downey, 1991, Gilbert et al., 2002, Kessler, 1997). Animal models of social stress involve exposure of a subject rodent to a conspecific. Chronic social defeat (where there is physical contact) and chronic social stress (where there is visual, auditory, and olfactory, but not tactile, contact) are models that rely on an ethological stressor and result in behavioural changes that mimic human depression (Huhman, 2006). Rodents exposed to repeated social defeat exhibit reduced sucrose preference, deficits in social interaction, reduced body weight (Berton et al., 2006, Tsankova et al., 2006, Becker et al., 2008), impaired working memory and enhanced fear memory (Yu et al., 2011), and increased immobility in the forced swim test (Rygula et al., 2005, Becker et al., 2008). This model of depression has been shown to increase sensitivity to mechanical (Rivat et al., 2010, Kim et al., 2012) and thermal (Marcinkiewcz et al., 2009, Kim et al., 2012) stimuli in rats. In contrast, increased (Tramullas et al., 2012) and no change (Yu et al., 2011) in hot plate latencies are reported in mice. Species differences may account for this inconsistency. However, visceral hyperalgesia, demonstrated as increased responding and reduced thresholds to colorectal distension, has been shown following chronic social defeat and/or overcrowding in both mice (Tramullas et al., 2012) and rats (Vicario et al., 2012). Moreover, CFA-induced mechanical allodynia and thermal hyperalgesia, and formalin-induced inflammatory hyperalgesia are exacerbated following chronic psychosocial stress (Andre et al., 2005, Rivat et al., 2010, Kim et al., 2012). A limitation of this model is that it tends to be ineffective in females, as females do not exhibit strong dominant or aggressive behaviour (Martinez et al., 1998). Taken together, this type of chronic stress, which is considered to be one of the most severe laboratory stressors used in rodents (Koolhaas et al., 1997) appears to enhance responding to nociceptive, visceral and inflammatory stimuli.

The learned helplessness theory of depression is based on the hypothesis that depression is a learned response to uncontrollable environmental stress.
Application of an uncontrollable and unpredictable stressor such as inescapable shock leads to a helpless state in animals (Vollmayr and Henn, 2001), an effect reversed by chronic antidepressant treatment (Sherman et al., 1982, Martin et al., 1987). Helpless animals have lower body weight, exhibit agitated behaviour, have alterations in sleep patterns, and cognitive deficits (Henn and Vollmayr, 2005). This model demonstrates increased thresholds in the von Frey and Hargreaves test (radiant heat), indicating mechanical and heat hypoalgesia (Li et al., 2013). However, a disadvantage of this model is that it cannot reliably be used to determine alterations in models of chronic pain, as the foot-shock is applied to the site of hypersensitivity, and therefore may confound results. Rodents that exhibit high levels of learned helplessness can be selectively bred, and the offspring of these animals (congenital learned helplessness) also exhibit increased thermal thresholds in the tail immersion test following shock-stress (King et al., 2001).

1.3.2 Early-life stress

Psychological trauma and childhood abuse frequently results in mood disorders in later life (Levitan and Parikh, 2003, Nemeroff, 2004b, Penza et al., 2003) and has also been reported as a predisposing factor to chronic pain in humans (Davis et al., 2005). Childhood abuse results in increased experimental pain sensitivity, particularly in females (Fillingim and Edwards, 2005). Early life stress in rodents is a clinically relevant model which has provided a vast literature on alterations in neurobiological systems mediating depressive- and anxiety-like behaviour (Pryce et al., 2002), but considerably less research has examined alterations in nociceptive responding. Early life stress results in reduced sensitivity (Coutinho et al., 2002, Weaver et al., 2007), no change (Uhelski and Fuchs, 2010), and increased sensitivity (Stephan et al., 2002, Imanaka et al., 2008) to thermal stimuli. Discrepancies between studies may be due to different strains (Fisher vs. Lewis) or gender (Stephan et al., 2002) or the type/duration of stressor (neonatal isolation vs. brief maternal separation) (Imanaka et al., 2008, Uhelski and Fuchs, 2010). In addition, increased sensitivity to mechanical stimuli is reported in some (Green et al., 2011,
Alvarez et al., 2013) but not all studies (Uhelski and Fuchs, 2010), which may relate to the site of application of the noxious stimulus. For example, mechanical hyperalgesia of the muscle (Green et al., 2011, Alvarez et al., 2013) but not skin (Uhelski and Fuchs, 2010, Green et al., 2011) has been reported following early life stress. Maternally separated animals display increased avoidance of a noxious mechanical stimulus, suggesting increased affective pain behaviour (Uhelski and Fuchs, 2010) and sound-stress in adulthood exacerbates mechanical hyperalgesia in the neonatal limited bedding model (Alvarez et al., 2013), suggesting interactions between stress, affect and pain following early life stress. It is consistently reported that visceral hypersensitivity occurs in adulthood following repeated maternal separation (3–6hrs in the first 2 weeks of life) (O'Malley et al., 2010, Coutinho et al., 2002, Slotten et al., 2006, O'Mahony et al., 2009, O'Mahony et al., 2011, Tsang et al., 2012, Chung et al., 2007a, Bian et al., 2010, Gosselin et al., 2010a, Moloney et al., 2012, Wouters et al., 2012), making it an excellent model for the study of mechanisms relating to irritable bowel syndrome, a condition frequently comorbid with depression and anxiety. Moreover, rats exposed to early life stress exhibit enhanced inflammatory hyperalgesia in the formalin test of persistent inflammatory nociception (Uhelski and Fuchs, 2010) and prolonged prostaglandin E₂–induced mechanical hyperalgesia (Green et al., 2011). Thus, early life stress results in altered (increased/decreased) nociceptive responding, with a reliable manifestation of visceral hypersensitivity and increased inflammatory nociceptive responding.

1.3.3 Pharmacological and immunological based animal models of depression

The monoamines play a central role in the relationship between depression and pain, which will be discussed in greater detail later in this chapter. Reserpine is an antihypertensive drug that depletes monoamine neurotransmitters in the brain and induces depressive-like behaviour such as increased immobility in the forced swim test, anhedonia and also reduces locomotor activity (Skalisz et al., 2002, Arora et al., 2011, Huang et al., 2004, O'Neil and Moore, 2003). Moreover, reserpine treatment has been shown to result in altered nociceptive behaviour, depending on the dose,
stimulus, and time of testing. Acute reserpine treatment results in biphasic responding. Initially, reserpine results in thermal and mechanical hypoalgesia exemplified as increased tail-flick and hot-plate latencies and increased mechanical response thresholds 24 hours after injection (Bisong et al., 2011, Oe et al., 2010). In contrast, mechanical, cold and heat hyperalgesia/allodynia are manifest from day 2 up to day 21 after a single injection, depending on the stimulus modality (Oe et al., 2010). These effects are accompanied by diverse and divergent alterations in dopamine, serotonin, noradrenaline and their metabolites in the spinal cord, thalamus and PFC (Oe et al., 2010), suggesting that altered monoaminergic tone in different regions of the CNS may be implicated in influencing nociceptive thresholds in a stimulus-dependent manner (Oe et al., 2010). In addition, repeated reserpine treatment (1 mg/kg for 3 days) results in pronounced mechanical hyperalgesia and allodynia, and cold allodynia lasting up to 28 days post-treatment (Ogino et al., 2013, Nagakura et al., 2012) which is accompanied by increased immobility in the forced swim test (Nagakura et al., 2009, Arora et al., 2011, Xu et al., 2013). Due to the depressive- and pain-related behaviour exhibited in the model, it has been proposed that administration of reserpine to rodents may be useful as an animal model of fibromyalgia (Nagakura et al., 2009) or depression-pain comorbidity (Xu et al., 2013). Thus, these data suggest that reduced monoaminergic signalling plays a key role in the development of concomitant depression and pain.

Activation of the immune system or exogenous administration of cytokines induces “sickness behaviour” in humans and animals which resembles symptoms of depression: decreased social interaction, lethargy, changes in weight and food consumption, anhedonia, sleep disturbances, and cognitive deficits (Castanon et al., 2001, O’Connor et al., 2009b, Dantzer and Kelley, 2007, De La Garza, 2005, Dantzer, 2001, Kent et al., 1992). Following systemic administration of the toll-like receptor (TLR)-4 agonist lipopolysaccharide (LPS), rodents display these sickness behaviours, and after these behaviours resolve, depressive-like behaviour is observed (24 hours post-injection). LPS-induced depressive-like behaviour is reported as
increased immobility in the forced-swim test and tail suspension test, decreased consumption of a sucrose solution and a suppression of sexual behaviour, which can be attenuated by chronic antidepressant (fluoxetine, imipramine) and anti-inflammatory treatment (minocycline) (Dunn and Swiergiel, 2005, Yirmiya, 1996, Yirmiya et al., 2001, Frenois et al., 2007, O'Connor et al., 2009b). However, it must be noted that LPS can cause a generalised reduction in locomotor activity (Dunn and Swiergiel, 2005, Yirmiya, 1996, O'Connor et al., 2009b), which may confound tests such as the FST or TST, and indeed nociceptive tests based on reflexive responses.

The effects of LPS on nociceptive responding are well established. For example, intraplantar (Kaur et al., 2005, Raghavendra et al., 2000), intrathecal (Loram et al., 2011, Meller et al., 1994) and systemic (Abe et al., 2001, Yoon et al., 2012, Satyanarayana et al., 2004) administration of LPS has been shown to elicit thermal hyperalgesia (in the tail-flick, hot plate and Hargreaves tests), mechanical hyperalgesia and allodynia, and increases behavioural signs of hyperalgesia such as paw-licking. Systemic LPS has been shown to elicit persistent muscle mechanical hyperalgesia which endures for up to 7 days post-administration, an effect which is further exacerbated by exposure to unpredictable sound stress (Dina et al., 2011). Furthermore, intraperitoneal LPS induces visceral hypersensitivity (Coelho et al., 2000b, Coelho et al., 2000a, O’Mahony et al., 2013). LPS potentiates formalin-induced nociceptive behaviour when administered 12 and 16h prior to testing (Padi and Kulkarni, 2005). A single systemic injection of LPS induces mechanical hyperalgesia, however repeated administration results in tolerance to LPS and a loss of the hyperalgesic response (Guo and Schluesener, 2006), thus this model may be preferable to examine nociceptive behaviours in a more acute, rather than chronic, timeframe.

LPS appears to exert a differential temporal effect on depressive-like and nociceptive behaviours. Systemic LPS (100 μg/kg) resulted in reduced social exploration in both males and females, reduced locomotor activity in males, but immobility in the FST was unaltered at 2 hours post-treatment (Pitychoutis et al., 2009). In addition, hot plate latencies were significantly increased at 2 hours post-LPS in both males and females (Pitychoutis et al., 2009). The nociceptive response to LPS appears to be biphasic.
Administration of LPS (200 μg/kg) resulted in thermal hyperalgesia in the hot plate test 30 min post-treatment, but resulted in hypoalgesia from 2 hours after and was abolished by 30 hours (Yirmiya et al., 1994). Conversely, depressive-like behaviour begins around 6 hours in the forced swim and tail suspension tests and remains at 24 hours post-LPS using a higher dose of 830 μg/kg (Frenois et al., 2007). These data suggest that acute activation of the immune system elicits time- and dose-dependent effects on nociceptive responding and depressive-like behaviour. However, the relationship between pain and depression in this model remains to be clarified.

1.3.4 Models based on a genetic predisposition to depressive-like behaviour

As genetics can confer a vulnerability to the development of depressive disorders, it is of key importance to investigate nociception in genetic animal models of depression. The Wistar-Kyoto (WKY) rat is a stress hyperresponsive rat strain which exhibits depressive- and anxiety-like behaviours such as increased immobility in the FST and reduced time in the inner zone of the open field and in the open arms of the EPM (Gentsch et al., 1987, Malkesman and Weller, 2009, Tejani-Butt et al., 1994). Work from our laboratory has demonstrated that WKY rats exhibit thermal hyperalgesia in the hot plate, but not tail flick test (Burke et al., 2010), and while our data failed to show any change in nociceptive responding to mechanical stimuli in these animals, mechanical allodynia has been reported previously (Taylor et al., 2001). Several studies have demonstrated visceral hyperalgesia to colorectal distension in the WKY rat (Gibney et al., 2009, Gosselin et al., 2010a, O'Malley et al., 2010, Gunter et al., 2000, Martinez et al., 2007, O’ Mahony et al., 2013, McKernan et al., 2010). In addition, water avoidance stress resulted in enhanced urinary bladder hyperalgesia in WKY rats (Robbins et al., 2007). We have reported enhanced inflammatory hyperalgesia in the formalin test, when compared to Sprague Dawley controls (Burke et al., 2010) and mechanical hyperalgesia was exacerbated in WKY rats following CFA administration to the temporomandibular joint (Wang et al., 2012a). Following peripheral nerve injury (CCI), WKY rats exhibit exacerbated mechanical allodynia compared to Wistar controls.
(Zeng et al., 2008b). However, contradicting reports in relation to inflammatory nociceptive responding have been reported. LPS-induced visceral hypersensitivity was blunted in WKY rats (O’Mahony et al., 2013) and the response to intraplantar zymosan administration was also blunted (Taylor et al., 2001) when compared to Sprague Dawley rats, indicating that different inflammatory stimuli elicit divergent effects on nociceptive responding in this model. Thus, stress hyperresponsivity of a genetic model of depression is associated with altered nociceptive, inflammatory and neuropathic pain behaviour depending on the stimulus/model examined.

While the WKY rat is considered a model of depression and comorbid anxiety (Tejani-Butt et al., 2003), the Flinders Sensitive Line (FSL) does not exhibit anxiety-like behaviour and as such are considered a true genetic model of depression (Braw et al., 2006). Flinders Sensitive Line rats demonstrate reduced locomotor activity, lower body weight, increased immobility in the FST, reduced sucrose preference, altered sleep patterns and cognitive deficits (Malkesman and Weller, 2009, Overstreet, 2002, Overstreet et al., 2005, Wallis et al., 1988). These rats exhibit cholinergic, serotonergic, and dopaminergic alterations (Overstreet et al., 2005). FSL rats do not exhibit significantly altered mechanical or thermal responding when compared to SD rats. However, FSL rats demonstrate reduced responding to mechanical and thermal stimuli following partial sciatic nerve ligation (Shir et al., 2001) and a higher pain threshold to electric shock compared to Flinders resistant line (Pucilowski et al., 1990). Thus, genetic susceptibility to depression alters nociceptive responding depending on the model under investigation, suggesting that the underlying neurobiological mechanisms in different genetic models may impact on pain pathways in a divergent manner.

1.3.5 Lesion model of depression

The olfactory bulbectomised (OB) rat is a lesion model of depression, involving removal of the olfactory bulbs which leads to neuronal reorganisation in cortico-limbic regions resulting behavioural, immunological, neuroendocrine and neurochemical changes that mimic the
symptoms of depression in humans (for review see Kelly et al., 1997, Song and Leonard, 2005). OB rodents exhibit hyperactivity in the open field, anhedonia and increased immobility in the FST (Roche et al., 2007, Romeas et al., 2009, Stock et al., 2000, Rinwa et al., 2013, Roche et al., 2008), impairments in learning and memory (Jaako-Movits and Zharkovsky, 2005, Nakagawasai et al., 2003), and reduced social behaviour (Liebenauer and Slotnick, 1996). The most consistent behavioural change caused by bulbectomy is the hyperactive response in a novel, brightly lit open field apparatus, which is reversed selectively by chronic, but not acute, antidepressant treatment (Cryan et al., 1998, Kelly et al., 1997). Thus, this model remains the best currently available for detecting antidepressant activity (Cryan et al., 2002, Kelly et al., 1997, Song and Leonard, 2005). Recently, we have demonstrated that OB rats exhibit mechanical allodynia and transient thermal hyperalgesia (Burke et al., 2010). OB rats have been shown to exhibit increased sensitivity to acute mechanical and thermal stimuli in the paw pressure test and tail-flick test, respectively (Rodríguez-Gaztelumendi et al., 2006). However, contradictory data of increased thermal and mechanical thresholds have also been reported (Wang et al., 2010b, Belcheva et al., 2009, Su et al., 2010), effects that may be dependent on the type, intensity and duration of stimuli applied. In addition, foot-shock thresholds have been reported to be unchanged in the model (van Riezen et al., 1977). In tests of persistent nociception, hyperalgesia to an inflammatory stimulus in the formalin test is enhanced in OB rats (Burke et al., 2010, Wang et al., 2010b). The OB rat demonstrated exacerbated nociceptive behaviours in response to electrical stimulation of the dura mater surrounding the superior sagittal sinus, a model of trigeminovascular nociception (Liang et al., 2011). Furthermore, radiation-induced analgesia to intraperitoneal acetic acid is completely abolished in OB mice (Miyachi, 1997). Thus, although responses to acute stimuli are increased/decreased depending on the test employed, all studies concur that OB rats exhibit enhanced nociceptive responding to persistent inflammatory or neural stimulation (Liang et al., 2011, Burke et al., 2010, Wang et al., 2010b).
<table>
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<tr>
<th>Model</th>
<th>Test</th>
<th>Response</th>
<th>Nociceptive behaviour</th>
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<tr>
<td>Unpredictable chronic mild stress</td>
<td>HP</td>
<td>↑ latency</td>
<td>Thermal hypoalgesia</td>
<td>(Shi et al., 2010a, 2010b, Qi et al., 2013)</td>
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<tr>
<td></td>
<td>HG</td>
<td>↑ latency</td>
<td></td>
<td>(Sacharczuk et al., 2009)</td>
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<tr>
<td></td>
<td>HP</td>
<td>↓ thresholds</td>
<td>Thermal hyperalgesia (in high swim-stress induced analgesia)</td>
<td>(Shi et al., 2010b, Qi et al., 2013, Shi et al., 2010b)</td>
</tr>
<tr>
<td></td>
<td>Electrical stimulation</td>
<td>↑ responding</td>
<td>Enhanced nociceptive behaviour</td>
<td>(Zhang et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>HP+CFA</td>
<td>↑ latency</td>
<td>Thermal hypoalgesia</td>
<td>(Shi et al., 2010b)</td>
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<tr>
<td></td>
<td>Formalin</td>
<td>↑ responding</td>
<td>Inflammatory hyperalgesia</td>
<td>(Qi et al., 2013, Shi et al., 2010b)</td>
</tr>
<tr>
<td></td>
<td>VF+SNL</td>
<td>↑ thresholds</td>
<td>Decreased sensitivity to mechanical and thermal stimuli after SNL</td>
<td>(Shi et al., 2010b)</td>
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<tr>
<td></td>
<td>HP+SNL</td>
<td>↑ thresholds</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>AT+CCI</td>
<td>↑ responding</td>
<td>Bilateral cold allodynia following CCI/sham surgery</td>
<td>(Bravo et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>CCI+PEAP</td>
<td>↑ responding</td>
<td>Increased escape/avoidance of a noxious mechanical stimulus</td>
<td>(Bravo et al., 2012)</td>
</tr>
<tr>
<td>Chronic unpredictable stress</td>
<td>TF</td>
<td>↑ thresholds</td>
<td>Thermal hypoalgesia</td>
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<tr>
<td>Chronic predictable stress</td>
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<td>Bilateral mechanical allodynia</td>
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<tr>
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<td>Mechanical allodynia and/or hyperalgesia</td>
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<tr>
<td></td>
<td>HP</td>
<td>↓ thresholds</td>
<td>Thermal hyperalgesia</td>
<td>(Spezia Adachi et al., 2012)</td>
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<td></td>
<td>Tail flick</td>
<td>↓ thresholds</td>
<td>Thermal hyperalgesia</td>
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<td></td>
<td>Tail immersion (at 42°C only)</td>
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<tr>
<td>Chronic social defeat stress</td>
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<td></td>
<td>Paw pressure</td>
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</tr>
<tr>
<td></td>
<td>HP</td>
<td>↑ thresholds</td>
<td>Thermal hypoalgesia</td>
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<td>↑ responding and ↓ thresholds</td>
<td>Visceral hyperalgesia</td>
<td>(Marcinkiewcz et al., 2009)</td>
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<td>Formalin</td>
<td>↑ responding</td>
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<td><strong>Chronic social stress</strong></td>
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<tr>
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<td>CFA+HG</td>
<td>↓ thresholds</td>
<td>Similar thresholds to non-isolated increased escape/avoidance of a noxious mechanical stimulus</td>
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<td>CCI+VF+AT</td>
<td>-</td>
<td>Increased escape/avoidance of a noxious mechanical stimulus</td>
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<td>CFA+VF/HP</td>
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<td>↓ thresholds</td>
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<td>(Green et al., 2011)</td>
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<td>Exacerbated mechanical hyperalgesia following sound stress</td>
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### Neonatal isolation

<table>
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<td>HP</td>
<td>↑ thresholds</td>
<td>Thermal hypoalgesia</td>
<td>(Imanaka et al., 2008)</td>
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### Chronic Reserpine

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<tr>
<td>VF</td>
<td>↓ thresholds</td>
<td>Mechanical allodynia</td>
<td>(Arora et al., 2011, Nagakura et al., 2012) (Xu et al., 2013).</td>
</tr>
<tr>
<td>Paw and muscle pressure</td>
<td>↓ thresholds</td>
<td>Mechanical hyperalgesia</td>
<td>(Nagakura et al., 2009, Arora et al., 2011, Ogino et al., 2013, Nagakura et al., 2012 (Nagakura et al., 2012).</td>
</tr>
<tr>
<td>AT</td>
<td>↑ responding</td>
<td>Cold allodynia</td>
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### Acute reserpine

<table>
<thead>
<tr>
<th>Condition</th>
<th>Response</th>
<th>Effect</th>
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</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td>↑ thresholds</td>
<td>Thermal hypoalgesia (24 h)</td>
<td>(Bisong et al., 2011)</td>
</tr>
<tr>
<td>TF</td>
<td>↑ thresholds</td>
<td>Thermal hyperalgesia (2-4 h, 3-5 days)</td>
<td>(Bisong et al., 2011)</td>
</tr>
<tr>
<td>HG</td>
<td>↓ thresholds</td>
<td>Cold allodynia (2-10 days)</td>
<td>(Oe et al., 2010)</td>
</tr>
<tr>
<td>AT</td>
<td>↑ responding</td>
<td>Mechanical allodynia (2-21 days)</td>
<td>(Oe et al., 2010)</td>
</tr>
<tr>
<td>VF</td>
<td>↑ thresholds</td>
<td>Mechanical hypoaiglesia (2-24 h)</td>
<td>(Oe et al., 2010)</td>
</tr>
<tr>
<td>Muscle pressure</td>
<td>↑↓ thresholds</td>
<td>Mechanical hyperalgesia (2-10 days)</td>
<td>(Oe et al., 2010)</td>
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### Inflammation-induced depression

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<th>Response</th>
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<tbody>
<tr>
<td>Electronic VF</td>
<td>↓ thresholds</td>
<td>Mechanical hyperalgesia</td>
<td>(Guo and Schluesener, 2006)</td>
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<tr>
<td>Muscle Pressure</td>
<td>↓ thresholds</td>
<td>Muscle hyperalgesia, exacerbated by unpredictable sound stress</td>
<td>(Dina et al., 2011)</td>
</tr>
<tr>
<td>HP</td>
<td>↑ thresholds</td>
<td>Thermal hypoalgesia</td>
<td>(Pitychoutis et al., 2009)</td>
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### Wistar Kyoto

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<th>Response</th>
<th>Effect</th>
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<tbody>
<tr>
<td>VF</td>
<td>↓ thresholds</td>
<td>Mechanical allodynia</td>
<td>(Taylor et al., 2001)</td>
</tr>
<tr>
<td>HP</td>
<td>↓ thresholds</td>
<td>Thermal hyperalgesia</td>
<td>(Burke et al., 2010)</td>
</tr>
<tr>
<td>TF</td>
<td>no change</td>
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<td>(Taylor et al., 2001, Burke et al., 2010)</td>
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### CRD

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<th>Effect</th>
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</thead>
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<tr>
<td>CRD</td>
<td>↑ responding and ↓ thresholds</td>
<td>Visceral hyperalgesia</td>
<td>(Gibney et al., 2009, Gosselin et al., 2010a, O’Malley et al., 2010, Gunter et al., 2000, Martinez et al., 2007, McKernan et al., 2010) (O’Mahony et al., 2013).</td>
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### Zymosan

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<tbody>
<tr>
<td>LPS+CRD</td>
<td>↓ responding</td>
<td>Blunted LPS-induced visceral hyperalgesia</td>
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<tr>
<td>Zymosan</td>
<td>↓ response</td>
<td>Blunted mechanical and thermal response to intraplantar zymosan</td>
<td>(Taylor et al., 2001)</td>
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<tr>
<td>CFA+VF</td>
<td>↑ responding</td>
<td>Exacerbated mechanical</td>
<td>(Wang et al.,)</td>
</tr>
<tr>
<td>Procedure</td>
<td>Effect</td>
<td>Description</td>
<td>Reference</td>
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</tr>
<tr>
<td>Formalin</td>
<td>↑ responding</td>
<td>Inflammatory hyperalgesia after CFA-induced inflammation of the tempromandibular joint</td>
<td>(Burke et al., 2010)</td>
</tr>
<tr>
<td>VF+CCI</td>
<td>↓ threshold</td>
<td>Exacerbated mechanical allodynia following nerve injury</td>
<td>(Zeng et al., 2008b)</td>
</tr>
<tr>
<td>VF Heat (CO₂ laser)</td>
<td>No change</td>
<td>No change</td>
<td>(Shir et al., 2001)</td>
</tr>
<tr>
<td>VF+pSNL Heat+pSNL</td>
<td>↑ threshold</td>
<td>Decreased responding to mechanical and thermal following nerve injury</td>
<td>(Shir et al., 2001)</td>
</tr>
<tr>
<td>Foot shock</td>
<td>↑ threshold</td>
<td>Higher pain threshold to shock</td>
<td>(Pucilowski et al., 1990)</td>
</tr>
<tr>
<td>VF Paw pressure</td>
<td>↓ threshold</td>
<td>Mechanical allodynia</td>
<td>(Burke et al., 2010, Rodríguez-Gaztelumendi et al., 2006)</td>
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<td>Paw pressure</td>
<td>↑ threshold</td>
<td>Mechanical hypoalgesia</td>
<td>(Belcheva et al., 2009)</td>
</tr>
<tr>
<td>TF</td>
<td>↓ thresholds no change</td>
<td>Thermal hyperalgesia</td>
<td>(Rodríguez-Gaztelumendi et al., 2006)</td>
</tr>
<tr>
<td>HP</td>
<td>↓ threshold</td>
<td>Thermal hyperalgesia (transient)</td>
<td>(Burke et al., 2010)</td>
</tr>
<tr>
<td>HG</td>
<td>↑ threshold</td>
<td>Thermal hypoalgesia</td>
<td>(Wang et al., 2010b, Su et al., 2010)</td>
</tr>
<tr>
<td>Foot shock</td>
<td>No change</td>
<td>No change</td>
<td>(van Riezen et al., 1977)</td>
</tr>
<tr>
<td>Electrical stimulation</td>
<td>↑ responding</td>
<td>Increased trigeminovascular nociceptive behaviour</td>
<td>(Liang et al., 2011)</td>
</tr>
<tr>
<td>Formalin</td>
<td>↑ responding</td>
<td>Inflammatory hyperalgesia</td>
<td>(Burke et al., 2010, Wang et al., 2010b)</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>↑ responding</td>
<td>Blunted radiation-induced analgesia to formalin and acetic acid</td>
<td>(Miyachi, 1997)</td>
</tr>
</tbody>
</table>

Table 1.4 Altered nociceptive responding in models of depression. AT acetone test, CCI Chronic constriction injury, CFA complete Freund's adjuvant, CRD colorectal distension, HP hot plate, HG Hargreaves, LPS lipopolysaccharide, pSNL partial sciatic nerve ligation, PEAP Place escape/avoidance paradigm, PGE₂ prostaglandin E₂, SNL spinal nerve ligation, TF tail-flick, VF von Frey.
1.4 Shared substrates in pain and depression

A number of substrates and pathophysiological mechanisms have been proposed to account for the association between depression and pain. Discussed below is evidence indicating a possible role for specific neuroanatomical areas, the hypothalamic-pituitary-adrenal (HPA) axis, monoaminergic and other neurotransmitter systems, neurotrophins and inflammatory mediators in the interaction between depression and pain.

1.4.1 Neuroanatomical substrates underlying emotional and nociceptive processing

Supraspinal structures and central reorganization are integral in the maintenance of maladaptive chronic pain disorders and modulation of emotional responding (Apkarian et al., 2011, Baliki et al., 2006), providing a neural correlate for the high co-occurrence of depression and pain disorders. Herein, pain- and depression-related alterations in function and/or structure of the regions most commonly implicated in the interaction between emotion and nociception, namely the thalamus, prefrontal cortex (PFC), anterior cingulate cortex (ACC), insula, amygdala and hippocampus are discussed (Fig 1.3).
The thalamus, the relay centre of the brain, is a critical region in nociceptive processing, as the spinothalamic pathway terminates in the ventral posterior lateral thalamus and processes sensory/discriminative aspects of pain, while the medial thalamus assigns affective/emotional significance to pain. Neuroimaging studies have revealed that the volume of the thalamus is reduced in depression (Kim et al., 2008) and it is well known that damage to the thalamus can lead to thalamic syndrome, comprising of hyperalgesia and allodynia and emotional disturbances such as depression (Henry et al., 2008b). Positron emission tomography scans revealed a reduction of cerebral blood flow to the contralateral thalamus in neuropathic pain patients (Iadarola et al., 1995, Hsieh et al., 1995) and decreased grey matter density in the thalamus in chronic back pain patients (Apkarian et al., 2004). In addition, increased experimental thermal pain thresholds in depressed patients were associated with increased activation of the ventrolateral thalamus compared to healthy patients (Bar et al., 2007), an effect also observed in response to thermal pain following sad-mood induction in healthy subjects (Wagner et al., 2009). Depression and chronic pain therefore are associated with compromised structure and function of the
thalamus, which may result in enhanced nociceptive input to cortical and limbic regions.

The prefrontal cortex (PFC) plays a central role in emotion, showing consistently altered structure and function in depression as revealed by imaging and histological studies (Drevets et al., 1997, Cotter et al., 2002, Hastings et al., 2004), and clinical studies demonstrate an important role for this region in processing/modulating the affective and sensory components of pain, as determined by imaging, neuroelectrical and neurochemical methods (Apkarian et al., 2005, Lorenz et al., 2003). Grey matter density of the PFC is reportedly reduced in chronic back pain patients, an effect which correlated with duration, intensity, sensory and negative affective components of pain (Apkarian et al., 2004). The magnitude of cortical atrophy in these patients was remarkably equivalent to 10-20 years of normal aging. When the pain in these patients was neuropathic in origin, it was associated with even stronger negative affect and greater reduction in grey matter density (Apkarian et al., 2004). With regards to pain processing in depression, depressed patients have been shown to exhibit increased activation of the frontal cortex in response to painful pressure compared to patients exposed to sham treatment (Graff-Guerrero et al., 2008), and increased activation of the ventrolateral, dorsolateral and medial PFC in response to noxious thermal stimulation compared to healthy subjects (Lopez-Sola et al., 2010, Bar et al., 2007), an effect associated with higher heat pain thresholds (Bar et al., 2007). However, one study has reported that depressed subjects failed to show increased activation within the dorsolateral PFC, in comparison to that observed in control subjects in response to painful heat stimuli (Strigo et al., 2008a). Differences in activation in the dorsolateral PFC between the two studies [(Bar et al., 2007) and (Strigo et al., 2008a)] may relate to differences in experimental design. For example, noxious thermal heat was applied to the left (Strigo et al., 2008a) and right (Bar et al., 2007) volar wrist in the respective studies, and increased heat pain thresholds have been observed on the right, but not left (Bar et al., 2003). The PFC also plays a key role in mediating emotional responding in chronic pain patients. Depression scores correlated with
activation of the PFC in response to colorectal distension in patients with irritable bowel syndrome (Elsenbruch et al., 2009) and in response to evoked joint pain in rheumatoid arthritis patients (Schweinhardt et al., 2008). Further correlation analysis revealed that the medial PFC partially mediated the relationship between the effect of depression on clinical pain in rheumatoid arthritis (Schweinhardt et al., 2008) and PFC activation correlated with clinical pain scores in fibromyalgia patients with comorbid depression (Giesecke et al., 2005). These clinical data are supported by preclinical evidence for the PFC in the interaction between emotion and pain. In the spared nerve injury model of neuropathic pain, decreases in PFC volume were correlated with the onset of anxiety-like behaviour (Seminowicz et al., 2009). Inhibition of the medial PFC using microinjection of the GABA_A agonist, muscimol, further augmented the enhanced inflammatory hyperalgesia exhibited by UCMS rats in the formalin test of persistent tonic nociception (Qi et al., 2013). Thus, it appears that the PFC has an important role to play in the interaction between depression/emotion and pain.

The anterior cingulate cortex (ACC), a region involved in modulation of emotional and attentional information, has also been shown to exhibit alterations in depression and pain patients. For example, increased metabolism and decreased volume of ACC has been reported in depressed patients compared to healthy controls (Drevets et al., 2008, Drevets, 2001). This area is important in perception of pain intensity and pain-related attention (Davis et al., 1997) and is activated in response to acute pain (Treede et al., 1999, Casey, 1999) and activity is increased in chronic back pain patients (Baliki et al., 2006). This area has been shown to be activated in response to noxious stimulation in depressed patients. Increased activation of the ACC of depressed patients subjected to pressure pain was reported compared to patients who were exposed to sham treatment (Graff-Guerrero et al., 2008). Depressed subjects do not exhibit deactivation of the ACC as seen in healthy controls in response to painful thermal stimulation (Strigo et al., 2008a, Lopez-Sola et al., 2010). In clinical pain populations,
activation of the ACC correlated positively with clinical pain scores in fibromyalgia patients with depression (Giesecke et al., 2005) and anxiety symptoms in patients with irritable bowel syndrome were associated with pain-induced activation of the ACC (Elsenbruch et al., 2009). Preclinical animal studies have shown that the ACC is implicated in affective, but not sensory, nociception, as lesions of this area reverse formalin-induced conditioned place aversion but not nociceptive behaviour (Johansen et al., 2001). Furthermore, stimulation of the ACC reverses avoidance behaviour in the place escape/avoidance paradigm in a model of neuropathic pain (LaBuda and Fuchs, 2005). Thus, impaired functioning of the ACC during pain processing in depressed patients may result in enhanced pain-related emotional responding.

The insular cortex plays a key role in integrating the sensory, affective and cognitive components of pain and emotion in humans (Damasio et al., 2000, Albanese et al., 2007, Coghill et al., 1999). In chronic back pain patients, duration of pain was strongly associated with increased activity in the insula (Baliki et al., 2006). In contrast, decreased activation of the insula in MDD subjects has been described which resolves following antidepressant treatment (Fitzgerald et al., 2008, Brody et al., 2001). However, it has been shown that depressed patients exhibit increased activation of the insula in response to painful pressure (Graff-Guerrero et al., 2008), in response to noxious thermal stimulation (Bar et al., 2007) and in anticipation of pain (Strigo et al., 2013). This latter effect was positively correlated with depressive symptom severity (Strigo et al., 2013). Activation of the insula of fibromyalgia patients in response to painful pressure also correlated with depression scores (Giesecke et al., 2005). Thus, the insular cortex appears to be one neuroanatomical site for the integration of emotional and pain processes.

The amygdala is a key part of the limbic system involved in the processing of emotional responses to stressors and the attachment of emotional value to pain and other sensory stimuli (Casey, 2006, Nemeroff, 2004a). Activity of the amygdala has been consistently demonstrated to be enhanced in clinical
depression (Peluso et al., 2009, van Wingen et al., 2010, Sheline et al., 2001, Drevets et al., 1992, Leppanen, 2006) and chronic pain (Geha et al., 2007, Kulkarni et al., 2007). Acute stress and fear are thought to reduce nociceptive responding by activating amygdala-linked inhibitory control systems and descending inhibitory pathways, switching off pain (Neugebauer et al., 2009). In contrast, chronic more persistent low-grade stress and stress-related disorders activate pain-facilitating pathways, switching on pain (Neugebauer et al., 2009) via corticotrophin-releasing factor, a neuropeptide involved in the stress response (Rouwette et al., 2012). This area is implicated in the link between pain and depression. For example, depressed patients exhibited increased activation of the amygdala in anticipation of and during the application of a painful heat stimulus when compared to healthy controls (Strigo et al., 2008a). Activation of the amygdala in response to painful pressure in fibromyalgia patients correlated with depression scores (Giesecke et al., 2005) and correlated with pain intensity ratings in cluster headache patients with comorbid depression (Seifert et al., 2010). Following sad mood induction, pain-related unpleasantness was associated with increased amygdala activation, linking negative emotion regulation with enhancement of pain affect (Berna et al., 2010). In preclinical models of chronic pain, depressive-like behaviour in the spared nerve injury model of neuropathic pain was accompanied by increased amygdala volume (Goncalves et al., 2008) and microinjection of the SSRI paroxetine into the basolateral amygdala reduced anxiety-related behaviour in sciatic nerve ligated mice (Matsuzawa-Yanagida et al., 2008). Inhibition of the basolateral amygdala using microinjection of the GABA_A agonist, muscimol, prevented the enhanced inflammatory hyperalgesia exhibited by UCMS rats in the formalin test of persistent tonic nociception (Qi et al., 2013). Thus, enhanced activity of the amygdala is critically linked to increased pain, affect and depression.

Another crucial limbic region in pain and depression is the hippocampus, a stress-vulnerable region important in mood, memory and learning. Reductions in hippocampal volume are reported in depression (Lange and
Irle, 2004, Weniger et al., 2006, Campbell et al., 2004) and in chronic back pain and complex regional pain syndrome (Mutso et al., 2012). Moreover, the hippocampus has been implicated in pain processing (Liu and Chen, 2009). The hippocampus has been shown to modulate the exacerbation of pain by anxiety in humans. Using fMRI, it was demonstrated that the hippocampus responded differentially to identical noxious thermal stimuli, dependent on whether the pain intensity was enhanced by anxiety, via connections with the cingulate and insular cortices (Ploghaus et al., 2001). Depressed subjects exhibited increased activation in the parahippocampal gyrus during painful stimulation when compared to control subjects (Strigo et al., 2008a) and greater activation of hippocampus in response to painful thermal stimulation when compared to healthy controls (Lopez-Sola et al., 2010).

None of the aforementioned regions act in isolation to modulate affect and/or nociceptive processes. Functional (Bar et al., 2007) and structural (Apkarian et al., 2004) alterations of thalamocortical pathways have been described in depressed patients and in patients with chronic pain. In addition, increased functional connectivity between the insula and posterior thalamus and decreased functional connectivity between the dorsal insula and the inferior frontal gyrus in response to painful heat in depressed patients has been described (Strigo et al., 2013). As mentioned, preclinical studies have shown that inhibition of the mPFC enhances formalin-induced pain behaviour, while inhibition of the amygdala reduces pain behaviour in the chronic mild stress model of depression (Qi et al., 2013). Antidepressant treatment has been shown to reverse pain-related functional alterations in the brain of depressed patients. For example, repetitive transcranial magnetic stimulation delivered to the left dorsolateral PFC reduced pain in depressed patients, which was not associated with an antidepressant effect (Avery et al., 2007). Chronic duloxetine (a serotonin-noradrenaline re-uptake inhibitor) treatment attenuated depression, an effect correlated with reductions in pain-related activation of the dorsolateral PFC and insula (Lopez-Sola et al., 2010). Similarly, following antidepressant treatment, the ACC and insula were no longer activated in depressed patients in response
to pain, but the frontal cortex was, suggesting that treatment may reduce affective processing mediated at the level of the ACC and insular cortex and enhance frontal modulation of pain processing (Graff-Guerrero et al., 2008). Taken together, depression and pain are associated with enhanced activity of thalamic, cortical and limbic structures involved in regulating nociceptive and emotional processes, effects which may be revered by successful antidepressant/analgesic treatment.

### 1.4.2 Hypothalamic-pituitary-adrenal (HPA) axis

The hypothalamic-pituitary-adrenal (HPA) axis is responsible for the neuroendocrine response to stress, an adaptive function that is critical for survival (Herman and Cullinan, 1997). Stress activates neurons of the paraventricular nucleus of the hypothalamus releasing corticotrophin releasing factor (CRF) into the hypophyseal portal system and travels to the anterior pituitary stimulating CRF-1 receptors thus inducing release of adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH acts upon the adrenal cortex to initiate synthesis and release of the glucocorticoids (cortisol/corticosterone) that bind to glucocorticoid receptors (GR) in the hypothalamus and pituitary, resulting in negative feedback (i.e. reduced CRF and ACTH release), preventing hyperdrive of the axis. Binding of glucocorticoids to GR in the hippocampus and PFC exerts an inhibitory effect, while GR activation in the amygdala exerts an excitatory influence, on HPA axis activity (Herman and Cullinan, 1997).

Blackburn-Munro et al. have examined the role of stress and the HPA axis in chronic pain and depression in two excellent reviews (Blackburn-Munro and Blackburn-Munro, 2001, Blackburn-Munro, 2004). Depression is associated with HPA axis hyperactivation, elevated basal glucocorticoid concentration, altered diurnal release of ACTH/cortisol, adrenal hypertrophy (Holsboer, 2000, Rubin et al., 1987, Owens and Nemeroff, 1993, Nemeroff et al., 1992, Plotsky et al., 1998) and reduced negative feedback of the HPA axis which occurs in approximately half of patients (Arana et al., 1985, Plotsky et al., 1998). In addition, successful antidepressant treatment often results in normalisation of the HPA axis response to stress (Himmerich et
Hypercortisolemia has also been reported in chronic pain. Higher cortisol levels are observed in patients with severe non-malignant chronic pain (Van Uum et al., 2008), rheumatoid arthritis (Catley et al., 2000), migraineurs (Peres et al., 2001), tempromandibular disorder (Korszun et al., 2002) and chronic back pain (Vachon-Presseau et al., 2013). However, conflicting findings of hypocortisolemia are reported in chronic whiplash-associated disorder (Gaab et al., 2005), chronic pelvic pain (Heim et al., 1999), and fibromyalgia (Lentjes et al., 1997, Crofford et al., 1994, Griep et al., 1998). Chronic pain patients may exhibit differential cortisol profiles depending on the pathophysiology or duration of the disease. Despite the discrepancies, both depression and chronic pain appear to be associated with a fundamental dysfunction of the HPA axis. With regards to the interaction between the HPA axis, depression and pain, it has been shown that patients with chronic low back pain had lower salivary cortisol than depressed patients and healthy controls, but experimental heat pain did not alter cortisol levels in any of the groups (Muhtz et al., 2013). When clomipramine was intravenously administered as a neuroendocrine challenge, depressed patients that exhibited low cortisol release showed increased heat pain thresholds compared to the high corticosterone response group (Kundermann et al., 2009). In comparison, higher serum cortisol has been shown to predict which individuals will develop chronic widespread pain in a group of psychologically at-risk subjects (McBeth et al., 2007) and a subset of fibromyalgia patients with depression exhibited higher cortisol levels (Wingenfeld et al., 2010). In addition, females with chronic pelvic pain and high depressive symptoms demonstrated enhanced cortisol response to ACTH stimulation compared to women with low depression scores (Wingenfeld et al., 2009) and plasma cortisol and ACTH was positively correlated with pain, depression and fatigue in cancer patients (Thornton et al., 2010). Thus, high cortisol in chronic pain conditions appears to be related to depression, and may be related to the development of chronic pain in depression.

As discussed earlier in this chapter, stress is associated with altered pain behaviour in animal models, suggesting that the HPA axis plays at least some part in the nociceptive response to stress. Central administration of
CRF enhances visceral hypersensitivity, peripheral CRF reduces the pain threshold to colorectal distension, and blocking CRF\(_1\) and CRF\(_2\) inhibits exogenous CRF- and acute stress-induced visceral hyperalgesia (Tache et al., 2004, Schwetz et al., 2004, Million et al., 2003). Chronic psychosocial stress-induced visceral hyperalgesia was accompanied by decreased dark phase plasma corticosterone levels and thymus weight, with increased adrenal weight (Tramullas et al., 2012) whereas chronic unpredictable stress induced-antinociception in the tail-flick test was accompanied by increased plasma corticosterone (Pinto-Ribeiro et al., 2004). Stress-induced formalin antinociception by exposure to an open elevated plus maze resulted in enhanced plasma corticosterone, however adrenalectomy did not prevent this attenuation of pain behaviour (Mendes-Gomes et al., 2011). Placebo analgesia, which was achieved by conditioning mice with morphine and cued chambers, elicited antinociception in the hot plate test, reduced immobility in the forced swim and tail suspension tests and decreased plasma corticosterone and ACTH (Guo et al., 2011). These studies indicate that activation of the HPA axis occurs in parallel to stress, pain and depressive-like behaviours. Mechanistic studies have shown that chronic unpredictable stress induced-antinociception is mirrored by administration of the synthetic corticosteroid, dexamethasone (Almeida et al., 2009). However, the combination of chronic mild stress and chronic constriction injury did not alter plasma corticosterone levels in rats (Bravo et al., 2012), and sciatic nerve cuff-induced anxiety- and depressive-like behaviour was not accompanied by alterations in basal or post-stress levels of corticosterone, thymus or adrenal weights (Yalcin et al., 2011). However, the combination of chronic restraint stress and spared nerve injury resulted in enhanced plasma corticosterone levels in mice compared to either experimental group alone, and inhibiting corticosterone synthesis with metyrapone for 2 weeks before spared nerve injury eliminated chronic stress-induced exacerbation of mechanical allodynia (Norman et al., 2010b). Moreover, acute stress-induced exacerbation of neuropathic pain in the spared nerve injury model is blocked by a GR antagonist and mimicked by exogenous administration of corticosterone to non-stressed animals.
(Alexander et al., 2009). Thus, glucocorticoids may be implicated in the development of persistent pain states, particularly in relation to stress-induced affective states, an effect which supports clinical findings (McBeth et al., 2007).

1.4.3 Neurotransmitters

The monoamines, namely noradrenaline, serotonin and dopamine, remain the most intensively investigated neurotransmitters implicated in regulation of emotion and nociception. Monoaminergic projections heavily innervate corticolimbic regions and relay through structures that attach emotional, motivational and cognitive meaning to pain, and to areas that modulate homeostatic responses to nociceptive input (Millan, 2002). Specifically, the serotonergic nuclei located in the raphe nucleus and the noradrenergic pathways arising from the locus coeruleus project to the frontal cortex to regulate mood, cognition and executive function; the limbic system to modulate emotions; and other targets to control movement, sleep, feeding, weight etc. In addition, serotonergic and noradrenergic pathways also descend to brainstem nuclei such as the RVM and to the dorsal horn of the spinal cord where they regulate nociceptive inputs. Thus, monoamines are uniquely positioned in cortical, limbic, brainstem and spinal cord to regulate emotional and nociceptive processes.

The monoamine hypothesis of depression has been the mainstay of depression research for over 40 years. This theory posits that serotonin and noradrenaline levels are depleted in depression, and that antidepressant treatments that increase levels of monoamines in the brain alleviate depressive symptoms. However, this theory does not account for the delay in treatment response, as monoamine levels are increased within hours, but therapeutic response requires weeks of treatment (Baldessarini, 1989). In addition, a high number of patients fail to achieve relief or remission from antidepressant treatment, and unwanted side effects can limit compliance. Nonetheless, despite limitations, pharmacological treatments aimed at increasing serotonergic and noradrenergic transmission remain the gold standard for treating depression. Apart from the efficacy of antidepressant
treatments that increase monoamines, there is also a wealth of evidence for dysfunction of this system in depression. For example, noradrenaline depletion results in a relapse of depression in patients who respond to noradrenaline reuptake inhibitors, depressed patients exhibit elevated noradrenaline in plasma and cerebrospinal fluid (CSF), increased $\alpha_2$- and $\beta$-adrenergic receptors in the postmortem brain, and altered locus coeruleus neuron density (for review see Ressler and Nemeroff, 2000, Delgado, 2000). Decreased noradrenaline release is reported in treatment-resistant subjects (Garcia-Segura et al., 1999). CSF levels of serotonin are low in depressed subjects, especially in suicidal patients (Csernansky and Sheline, 1993, Mann and Malone, 1997). Moreover, plasma serotonin levels are negatively correlated with suicidal behaviour in adolescent inpatients (Tyano et al., 2006) and 5-Hydroxyindoleacetic acid (5HIAA), the metabolite of serotonin, is low in depressed patients and victims of suicide (Delgado, 2000).

In addition to modulating emotion, serotonin and noradrenaline are major players in the endogenous pain control system, modulating the descending facilitatory/inhibitory pathways (Millan, 2002). Evidence suggests that monoaminergic dysfunction occurs in chronic pain patients. Lower levels of serotonin metabolites have been reported in the CSF of chronic pain patients (Almay et al., 1987, Bouckoms et al., 1992), with female pain patients demonstrating higher levels of 5HIAA (France et al., 1987). In patients with post-traumatic pain, CSF concentrations of noradrenaline and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) were lower compared to controls, and these levels correlated with affective symptoms (Cui et al., 2012). Preclinical animal studies have demonstrated that serotonin exerts anti- or pro-nociceptive effects depending on the type of receptor stimulated and its location, the duration of pain, and its pathophysiology. For example, activation of $5HT_{1A}$ receptors in the spinal cord elicits an antinociceptive effect (Mico et al., 2006, Bardin et al., 2003, Kayser et al., 2007), whereas pronociceptive effects are mediated by $5HT_{2A}$ and $5HT_{3}$ receptors (Rahman et al., 2004, Green et al., 2000, Sasaki et al., 2006, Kayser et al., 2007,
Suzuki et al., 2004). In addition, depletion of endogenous 5HT in the spinal cord attenuates mechanical and cold allodynia after SNL from post-operative day 7 on, but not at earlier time points (Rahman et al., 2006), suggesting that supraspinal serotonergic neurotransmission may play a role in the maintenance, but not development, of chronic pain states. In animal models of neuropathic pain, serotonin levels are decreased in pain-processing regions including the cerebral cortex, the ventrobasal thalamus, the raphe magnus nucleus, and the spinal cord (Goettl et al., 2002, Hains et al., 2001, Liu et al., 2010, Sandrini et al., 1997, Sounvoravong et al., 2004), and formalin induces increases in serotonin, noradrenalaine and their metabolites in the spinal cord (Omote et al., 1998), PFC, amygdala, and hippocampus (Burke et al., 2010). Noradrenaline exerts its primary antinociceptive effects via action on $\alpha_2$ adrenoreceptors in descending inhibitory pain pathways, where it reduces the sensitivity of dorsal horn neurons to noxious but not non-noxious stimuli (Fields, 1994, Mansikka et al., 1996, Meng et al., 1997). Chronic constriction injury has been shown to enhance noradrenaline signalling in the locus coeruleus (Alba-Delgado et al., 2013), increased spinal release of noradrenaline attenuates post-operative pain (Wang et al., 2008c), and blocking noradrenaline elicits mechanical and cold allodynia in the spinal nerve ligation model of neuropathic pain (Xu et al., 1999). Furthermore, enhancing noradrenergic tone results in antinociception in preclinical models of acute, visceral, inflammatory, osteoarthritic, neuropathic, diabetic and bone cancer pain (Whiteside et al., 2010) and noradrenaline is crucially important for the analgesic efficacy of antidepressant drugs (Rojas-Corrales et al., 2003, Mico et al., 2006, Bomholt et al., 2005a).

In addition to serotonin and noradrenaline, dopamine is implicated in mood and pain regulation. Dopamine has been shown to play a role in pain processing in the spinal cord (Jensen et al., 1984), the periaqueductal grey (Meyer et al., 2009, Flores et al., 2004), thalamus (Shyu et al., 1992), basal ganglia (Chudler and Dong, 1995), insular cortex (Burkey et al., 1999), cingulate cortex (Lopez-Avila et al., 2004), the putamen and medial temporal cortex (Hagelberg et al., 2002). Decreased dopamine has been
associated with pain symptoms in patients with Parkinson's disease (Brefel-Courbon et al., 2005) and nigrostriatal lesion in a rat model of Parkinson's disease inhibited nitroglycerin-induced hyperalgesia (Greco et al., 2008). Dopamine dysregulation may underlie depressive symptoms such as psychomotor retardation and decreased motivation (Brown and Gershon, 1993), while dopaminergic drugs (antipsychotics, anti-parkinsonian drugs, atypical antidepressants, psychostimulants) modify pain perception (Potvin et al., 2009). Fibromyalgia patients have disrupted dopamine transmission in the brainstem, thalamus and limbic system (Wood et al., 2007, Russell et al., 1992, Legangneux et al., 2001) and decrements in PFC dopamine levels have been associated with inflammatory pain-related cognitive impairment (Pais-Vieira et al., 2009) and anxiety-like behaviour (Espejo, 1997).

1.4.3.1 Monoamines in the interaction between clinical depression and pain

As monoaminergic alterations have been reported in both depression and chronic pain, it is probably not surprising that this neurotransmitter system would be involved, at least in part, in the association between these disorders. Thus, reduced monoamine levels and altered neurotransmission in cortical and limbic regions may be responsible for the emotional and affective aspects of depression and pain while deficits in levels in brainstem regions such as the RVM may result in a disinhibitory effect on nociceptive processing at the level of the spinal cord. Thus, the widespread and unspecific pain with no organic cause often reported in depression may arise from dysfunction of these pathways (Stahl and Briley, 2004), effects that may be reversed by successful antidepressant treatment. Convincing evidence arises due to the efficacy of monoaminergic-based antidepressants in the treatment of both depression and as a first-line treatment for chronic pain (Jann and Slade, 2007, Mico et al., 2006, Fava et al., 2004, Marangell et al., 2011, Ward et al., 1984, Kerns, 2010, Brecht et al., 2007). Dysregulation of serotonergic and noradrenergic neurotransmission therefore might provoke or enhance both disorders (Stahl and Briley, 2004). Despite the well-known role of the monoamines in depression and pain, there is a surprising lack of studies examining pathological alterations in this
system in the interaction between depression and pain. In depressed patients with pain, more severe pain was associated with lower platelet monoamine oxidase activity than the patients without pain or with mild pain, which may suggest increased activity of serotonergic systems (von Knorring et al., 1984). However, a study reported no correlation between monoamine levels, pain and depression (Ghia et al., 1981). Depression, but not chronic widespread pain, is associated with decreased serotonin levels (Paul-Savoie et al., 2011). Fibromyalgia has been associated with decreased levels of peripheral and cerebrospinal tryptophan (5HT precursor), 5HT and 5HIAA, and decreased platelet levels of the serotonin transporter (Bazzichi et al., 2006, Yunus et al., 1992, Legangneux et al., 2001, Wolfe et al., 1997, Maes et al., 2000). Tryptophan supplementation in healthy patients increased electrical pain tolerance and was associated with mood elevation (Seltzer et al., 1982). However, how peripheral levels of monoamines relate to central processing of pain and depression remains to be determined. Animal studies have provided support for a role of monoamines in the association between pain and depression. As previously discussed, repeated treatment with reserpine in rats causes a decrease in dopamine, noradrenaline, and serotonin in the spinal cord, thalamus, and PFC (Nagakura et al., 2009, Oe et al., 2010), which results in mechanical allodynia/hyperalgesia with concomitant depressive-like symptoms (Nagakura et al., 2009, Arora et al., 2011, Oe et al., 2010). Chronic restraint stress-induced thermal hyperalgesia was associated with increased tryptophan hydroxylase, an enzyme involved in serotonin synthesis, in the rostral ventromedial medulla (Imbe et al., 2004). We have shown that enhanced inflammatory hyperalgesia in two rat models of depression is associated with alterations in monoamines across discrete brain regions responsible for processing affect and nociception (Burke et al., 2010). Specifically, the olfactory bulbectomised rat, which has been shown to exhibit antidepressant-sensitive hyperactivity, has been proposed to model hyposerotonergic depression (Lumia et al., 1992). OB rats exhibit serotonergic deficits and decreased turnover in the frontal cortex, nucleus accumbens, hypothalamus, hippocampus, amygdala and mid-brain (Lumia et al., 1992, Song and Leonard, 1995, Redmond et al., 1997, Connor et al., 1999, Hellweg et al., 2007). OB rats exhibit enhanced
formalin-induced nociceptive responding and blunted formalin-induced increases in serotonin and/or 5HIAA in the PFC, increases in serotonin in the thalamus, and increases in 5HIAA and noradrenaline in the cerebellum (Burke et al., 2010). Furthermore, enhanced nociceptive responding in OB rats was correlated with reduced 5HIAA levels in the hippocampus and amygdala following intraplantar formalin. Wistar-Kyoto (WKY) rats, a genetic model of depression, exhibit enhanced nociceptive responding to a persistent inflammatory stimulus (Burke et al., 2010). In addition, WKY rats exhibited attenuated formalin-induced increases in noradrenaline in the hypothalamus, hippocampus, amygdala, thalamus and cerebellum when compared to Sprague Dawley controls. Moreover, enhanced nociceptive behaviour was correlated with blunted formalin-evoked increases in 5HT and 5HIAA in the hypothalamus of WKY rats (Burke et al., 2010). In a rat model of neuropathic pain (CCI), persistent nerve injury leads to depressive-like behaviour and noradrenergic impairment in the locus coeruleus (Alba-Delgado et al., 2013). Specifically, 28 days after CCI, increased immobility at the expense of climbing behaviour is observed in the forced swim test, an effect accompanied by increased bursting activity of locus coeruleus (LC) neurons, increased noradrenaline transporter levels and α2A-adrenoceptor in the LC, and a greater inhibitory effect on PFC noradrenaline release. Further supporting a role for monoamines in depression-pain comorbidity is data showing that drugs that enhance monoaminergic signalling can reduce pain and depressive-like behaviour. Administration of the SSRI fluoxetine attenuated chronic restraint stress-induced hyperalgesia in the formalin test in rats (Gameiro et al., 2006), while amitriptyline (TCA) treatment reversed CCI-induced allodynia and depressive-like behaviour (Jesse et al., 2010). In addition, chronic amitriptyline treatment reversed enhanced trigeminovascular nociceptive responding in the olfactory bulbectomised rat model of depression (Liang et al., 2011). These data suggest that depression-related allodynia and hyperalgesia may be mediated, at least in part, by disruptions of serotonin and noradrenaline signalling in discrete brain regions and in the spinal cord, and that antidepressants may target both pain and depressive-like behaviour.
1.4.4 Other Neurotransmitter systems: Opioids, endocannabinoids, GABA and glutamate

Increasing evidence highlights a role for other neurotransmitter systems such as the opioids, endocannabinoids, glutamate and gamma-aminobutyric acid (GABA) in the regulation of emotion, pain and the interaction between these states. In-depth analysis of this literature is beyond the scope of this thesis, however presented are some key studies highlighting the role of these systems in depression-pain interactions.

Activation of descending pain inhibitory pathways leads to endogenous opioid (endorphin) release in the dorsal horn of the spinal cord and profound analgesia (Furst, 1999), while opioid release in cortical and limbic areas attenuates emotional aspects of pain (Hebb et al., 2005, Frew and Drummond, 2007). Opioids are capable of eliciting mood-elevating effects and are implicated in the some of the analgesic effect of antidepressants (Valverde et al., 1994, Wattiez et al., 2011). Increased cold pain thresholds in depressed patients do not appear to be mediated by opioid mechanisms (Lautenbacher et al., 1994), however psychological distress in depressed subjects has been shown to inhibit cold pain responding via stress-evoked release of opioid peptides (Frew and Drummond, 2008). Preclinically, stress-induced hyperalgesia in socially defeated animals is mediated by the kappa opioid system (McLaughlin et al., 2006) and thermal hypoalgesia in maternally separated rats was associated with increased mu-opioid binding in the brain (Weaver et al., 2007). Persistent inflammatory or neuropathic pain-induced anxiogenic behaviour was associated with reduced opioid binding in the amygdala (Narita et al., 2006a), and chronic restraint stress-induced hyperalgesia was associated with an impaired antinociceptive effect of morphine (da Silva Torres et al., 2003, Gameiro et al., 2006). These studies suggest that alterations in the endogenous opioid system may, in part, underlie the emotional and nociceptive changes observed in chronic pain and stress-related disorders.

Recently, increasing focus is on the endogenous cannabinoid (endocannabinoid) system in the regulation of stress, pain and mood.
Modulation of the endocannabinoid system regulates pain (Graham et al., 2009, Hohmann and Suplita, 2006) and anxiety and depression (Vinod and Hungund, 2006, Finn, 2009). Cannabinoid ligands are antinociceptive in acute, inflammatory and neuropathic pain models (Finn and Chapman, 2004, Manzanares et al., 2006) and alterations in the endocannabinoid system are reported in models of affective disorders including maternal deprivation and olfactory bulbectomy (Marco et al., 2012, Eisenstein et al., 2010). Only one study to date has been published examining the role of this system in depression-pain interactions demonstrating that depression-like behaviour in rats with chronic constriction injury is reduced by selective blockade of the cannabinoid CB2 receptor (Hu et al., 2009).

Other neurotransmitters such as the inhibitory GABA and excitatory amino acid neurotransmitter, glutamate, are also well-recognised to be involved in pain and depression (for review see Mohler, 2012, Paul and Skolnick, 2003, Goudet et al., 2009). Recently, ketamine, an antagonist of the glutamate NMDA receptor, has been shown to be highly effective in treatment-resistant depression (Zarate et al., 2006). A recent case study reported that repeated ketamine injection resulted in improved mood and pain in a cancer patient (Zanicotti et al., 2012). Ketamine has also been shown to attenuate nerve-injury induced depressive-like behaviour (forced swim and sucrose preference test) in rats (Wang et al., 2011). Chronic restraint stress in rats resulted in mechanical hypersensitivity and reduced protein levels of the glutamate excitatory amino acid transporter 2 protein level in the periaqueductal grey (Imbe et al., 2012) and exacerbated mechanical hyperalgesia in WKY rats following inflammation was accompanied by upregulation of the NR1 subunit of the NMDA receptor in the trigeminal subnucleus caudalis (Wang et al., 2012a). Riluzole, a drug which enhances glutamate reuptake, reverses early-life stress-induced visceral hypersensitivity (Gosselin et al., 2010a) and NMDA receptor antagonism prevents stress-induced exacerbation of neuropathic pain (Alexander et al., 2009). Moreover, gabapentin, a GABA analogue, has been shown to attenuate pain-related anxiety-like behaviour in the EPM and open field in
the CCI model (Ceci et al., 2008, Gregoire et al., 2012, Rice et al., 2008). Further research is required to examine the effect of these mediators on the interaction between depression and pain but current findings suggest that modulation of GABA and glutamate and endocannabinoid neurotransmission may represent novel targets for the treatment of depression and/or chronic pain.

1.4.5 Neurotrophins

Neurotrophic factors, including nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin 3 and brain derived neurotrophic factor (BDNF), promote survival, growth and maintenance of neurons. Of the neurotrophins, the greatest amount of research has been conducted on examining the role of BDNF in the pathophysiology of depression and chronic pain. Serum BDNF levels are reduced in depressed patients (Karege et al., 2005a, Karege et al., 2002, Shimizu et al., 2003) and human postmortem studies have revealed that BDNF expression is reduced in the hippocampus and PFC of depressed patients who died by suicide (Dwivedi et al., 2003, Karege et al., 2005b). In addition, chronic antidepressant treatment increases serum and hippocampal levels of BDNF (Gervasoni et al., 2005, Shimizu et al., 2003, Gonul et al., 2005, Chen et al., 2001, Karege et al., 2005b). Preclinically, several animal models of depression have been shown to exhibit reduced BDNF expression such as chronic stress, social defeat, and maternal deprivation (for review see Duman and Monteggia, 2006), antidepressant treatments (electroconvulsive treatment, SSRI’s, SNRI’s and TCA’s) are known to enhance BDNF levels in rodents (Van Hoomissen et al., 2003, Nibuya et al., 1995, Chen et al., 2001) and administration of BDNF into the hippocampus produces antidepressant-like effects in learned helplessness and forced swim test models (Shirayama et al., 2002, Siuciak et al., 1997, Hoshaw et al., 2005). At a peripheral level, BDNF exerts primarily a pronociceptive effect as exogenous BDNF induces thermal hyperalgesia and mechanical allodynia (Shu and Mendell, 1999, Shu et al., 1999, Miki et al., 2000, Karchewski et al., 2002). BDNF is upregulated in the dorsal root ganglia and spinal cord of animal models of inflammatory and neuropathic pain (Obata and Noguchi,
blockade of BDNF produces antinociceptive effects (Zhou et al., 2011, Obata and Noguchi, 2006, Li et al., 2008) and BDNF contributes to 
central sensitization at the spinal level in the CCI model of neuropathy (Lu 
et al., 2007, Lu et al., 2009), thus BDNF may provide a novel target for 
analgesic treatments. However, chronic pain elicits opposing effects on 
BDNF levels spinaly vs. supraspinally. For example, persistent 
inflammatory pain in the complete Freund’s adjuvant model was 
accompanied by increased BDNF in the spinal cord but reduced BDNF and 
decreased neurogenesis in the dentate gyrus of the hippocampal formation 
(Duric and McCarson, 2006b, Duric and McCarson, 2007), effects reversed 
by chronic treatment with the tricyclic antidepressant imipramine (Duric and 
McCarson, 2006a). Moreover, the spared nerve injury and chronic 
constriction injury models of neuropathic pain exhibit decreased BDNF in 
the cingulum, striatum and hippocampus (Al-Amin et al., 2011). Thus, 
similar to that reported for depression, persistent pain is associated with 
reduced hippocampal BDNF, ultimately resulting in reduced neurogenesis 
in this region that regulates emotion, memory, pain and the stress response. 

Few studies have examined the role of neurotrophins in depression-pain 
interactions. Depressive-like behaviours observed in the CCI model of 
neuropathic pain were associated with reduced BDNF expression in the 
hippocampus (Hu et al., 2010). Furthermore, intracerebroventricular 
administration of 4-MC, a stimulator of BDNF synthesis, elicits an 
antinociceptive effect in the CCI model, with a concurrent reduction of 
depression-like behaviour (Fukuhara et al., 2011). Chronic pain-induced 
reductions of BDNF in areas such as the hippocampus may lead to 
emotional deficits such as depression, and targeting this reduction may 
successfully ameliorate both pain and depression. However, the effect of 
pain on BDNF appears to be region-dependent. Chronic restraint stress for 2 
weeks prior to peripheral nerve injury exacerbated mechanical alldynia and 
depressive-like behaviour, effects accompanied by increased BDNF gene 
expression in PAG (Norman et al., 2010b). BDNF mRNA is highly 
expressed within the PAG (Ceccatelli et al., 1991, Conner et al., 1997) and 
blockade of BDNF in the PAG inhibits hyperalgesia, whereas
microinjection of exogenous BDNF produces hyperalgesia and descending pain facilitation (Guo et al., 2006). Thus, increased nociceptive signals may result in enhanced BDNF expression in the spinal cord and brainstem which may be a protective mechanism, with a reduction of BDNF expression in stress-sensitive regions such as the hippocampus that may lead to depression associated with chronic pain. Further research is required to clarify the spatiotemporal role of BDNF in the relationship between depression and pain.
1.4.6 The immune system in depression-pain comorbidity

The immune system functions to protect the body against disease, however excessive inflammation can be detrimental leading to damaged tissue. Diseases such as diabetes, cardiovascular disease, depression and chronic pain conditions are associated with increased levels of circulating pro-inflammatory cytokines, indicating peripheral inflammation. However, increasing evidence indicates that central inflammation (neuroinflammation) is implicated in affective and chronic pain disorders. Glial cells are the immune cells of the central nervous system which play a supportive and protective role to neurons, and are involved in synaptic plasticity and development of the healthy brain. Glial cells express receptors for the monoamines, GABA and glutamate, neurotrophins, chemokines and cytokines (for review see Rajkowska and Miguel-Hidalgo, 2007). The glial cells include microglia, astrocytes and oligodendrocytes. Oligodendroglia are crucial for maintenance of axonal integrity (Nave and Trapp, 2008), release of neurotrophic factors (Zhang et al., 2006b) and are important for glutamate clearance in white matter (Pitt et al., 2003). Microglia are the resident macrophages of the CNS, constantly surveilling the environment for homeostatic deviations, whereas astrocytes are crucial for neurotransmitter uptake and degradation at the synaptic cleft, neuronal nutritional/metabolic support, and repair and regeneration. When microglia are activated, usually in response to a pathogen or neuronal debris, they upregulate surface markers (OX42, CD11b) and release cytokines. Pro-inflammatory cytokines in turn activate astrocytes resulting in upregulation of their surface proteins glial fibrillary acid protein (GFAP) and S100β and the further release of inflammatory mediators. As a result, enhanced release of proinflammatory cytokines and the expression of surface proteins are often used as biomarkers to detect glial activation (Tzeng et al., 1999). Under physiological conditions, microglia play a protective and supportive role, however in pathological circumstances they release proinflammatory cytokines, prostaglandins, glutamate, growth factors and reactive oxidative and nitrosative species, mediators which can impact on neuronal functioning. Within the brain, cytokines have diverse effects on glia and
neurons, can activate the HPA axis, alter neurogenesis and the metabolism of monoamines (Song and Wang, 2011, Dantzer et al., 1999, Raison et al., 2006, Miller, 2009) and thus elicit powerful effects on behaviour, cognition, pain and mood (Dray, 1995, Kronfol and Remick, 2000, Wilson et al., 2002). The neuroprotective or neurodegenerative effects of cytokines and immune mediators in the brain depend on their concentration, the receptor which they activate and the conditions under which they are released. In general, excessive, unrestrained proinflammatory activity leads to neuronal damage, apoptosis and neurodegeneration (Allan and Rothwell, 2001, Rothwell and Relton, 1993). An abundance of evidence now supports a role for glial cell dysregulation in the pathophysiology of chronic pain and psychiatric disorders, and glial and neuroimmune processes are considered possible mediators in the association between these disorders (Strouse, 2007).

1.4.6.1 Inflammation, depression, and pain – clinical evidence

A large amount of evidence exists to suggest that depression and chronic pain are associated with alterations of the immune system, as described in a number of excellent reviews (Leonard, 2010, Maes et al., 1993, Sluzewska et al., 1996, Maes et al., 1997, Smith, 1991, Raison et al., 2006, Dantzer et al., 2008, Miller et al., 2009a, Watkins et al., 2001, Wieseler-Frank et al., 2004, Marchand et al., 2005, Schomberg and Olson, 2012). A full examination of inflammatory changes in depression and pain is beyond the scope of this thesis, therefore, presented here is a brief overview of the evidence demonstrating glial activation and neuroimmune changes in depression and chronic pain. Postmortem brains of depressed patients are reported to have low glial cell density in the PFC (Rajkowska et al., 1999, Ongur et al., 1998, Cotter et al., 2002), amygdala (Bowley et al., 2002), and ACC (Cotter et al., 2001), although the subpopulation of glial cell was not determined in these studies. Glial reduction determined immunohistochemically in the amygdala and PFC was shown to be due to loss of oligodendrocytes (Hamidi et al., 2004, Uranova et al., 2004). Studies have demonstrated using immunohistochemistry that GFAP-labelled astrocytes are reduced by 75% in the PFC of young depressed patients.
(Miguel-Hidalgo et al., 2000), and by 20-30% in the ACC and orbitofrontal cortex (Gittins and Harrison, 2011, Miguel-Hidalgo et al., 2010). Despite these reductions in GFAP, detailed morphometric analysis revealed that fibrous astrocytes were larger and had longer and more ramified processes in the ACC of depressed suicide patients (Torres-Platas et al., 2011). In comparison to a loss of astrocytes and oligodendrocytes, microglial activation has been described in the PFC, ACC and thalamus of depressed patients who died by suicide (Steiner et al., 2008). Recent studies have indicated increased mRNA and protein levels of a number of cytokines in the PFC of depressed patients including interleukin 1-beta (IL-1β), IL-6, TNFα, IL-1α, IL-2, IL-10, IL-13, and interferon-gamma (IFNγ) (Shelton et al., 2011, Pandey et al., 2012, Tonelli et al., 2008).

In addition, there is some evidence for spinal and supraspinal glial changes in chronic pain. A single postmortem case study of a patient with long standing chronic regional pain syndrome reported significant activation of both microglia and astrocytes in the spinal cord localised mostly at the level of the initial injury (left gastrocnemius muscle, L4–S2 roots), but was also apparent throughout the spinal cord (Del Valle et al., 2009). Up to 90% of patients infected with HIV suffer from chronic pain, and in half of these patients no organic cause can be found (Parente et al., 1994, Thuluvath et al., 1991). HIV patients with chronic pain exhibited increased markers of astrocyte activation (GFAP and S100β), but not microglia, and increased expression of the pro-inflammatory mediators TNFα and IL-1β in the dorsal horn of the spinal cord when compared to patients without pain (Shi et al., 2012). Supraspinal glial activation has also been described using positron emission tomography and magnetic resonance spectroscopy which revealed increased glial activation in the thalamus but not somatosensory cortex of patients with limb denervation (Banati et al., 2001), and in the ACC (Widerstrom-Noga et al., 2013) and thalamus (Pattany et al., 2002) of spinal cord injury patients with neuropathic pain. Thus, although scant, current reports indicate some level of glial activation in the spinal cord and brain of chronic pain patients, results consistent with an extensive body of research in animals (for review see Austin and Moalem-Taylor, 2010).
Evidence for a role of inflammation in the pathogenesis of depression-pain comorbidity arises from clinical studies reporting that depression (Raison et al., 2006, Capuron et al., 2002b, Capuron et al., 2004, Majer et al., 2008, Wichers et al., 2007) and pain (Shakoor et al., 2010, Capuron et al., 2002a) are common side effects of chronic IFNα treatment in over 50% of patients. Somatic symptoms such as pain (headaches, body aches, joint pain) begin as early as two weeks on IFNα treatment, whereas depressive mood and cognitive dysfunction appear after 8 weeks in vulnerable individuals (Capuron et al., 2002a) (Fig. 1.4). The psychological symptoms respond earlier to antidepressant treatment than the somatic symptoms (Capuron et al., 2002a). Further evidence for a role of cytokines in the depression-pain relationship arises from genetic studies in cancer patients which revealed that an IL-4 single-nucleotide polymorphism was associated with high self-reported levels of both pain and depression (Illi et al., 2012). More research is required to determine genetic alterations in immune-related genes in the association between depression and pain.

**Fig 1.4 Temporal evolution of the symptoms induced by chronic interferon-alpha therapy.** Adapted from (Capuron et al., 2002a). The somatic/neurovegetative symptoms develop rapidly in almost every individual exposed to cytokines and persist for the duration of IFN-alpha therapy. These symptoms are minimally responsive to antidepressant treatment. In contrast, the mood and cognitive symptoms develop in vulnerable patients at later stages of IFN-alpha therapy (between weeks 8 and 12) and are highly responsive to antidepressant medication.
Relatively few clinical studies have evaluated if depressed patients with comorbid pain or vice versa, chronic pain patients with depression, exhibit alterations in immune function. Outpatients with major depressive disorder display higher serum levels of TNFα (but not IL-6) and increased pressure pain sensitivity when compared to healthy controls (Euteneuer et al., 2010). Moreover, the augmented levels of TNFα correlate significantly with reduced pain thresholds in women, but not men (Euteneuer et al., 2010). Conversely, in primary care patients with high depressive symptoms, increased plasma IL-6 was associated with greater bodily pain ratings (more pain intensity and interference), an effect not observed in patients with low depressive symptoms (Poleshuck et al., 2013), and patients with chronic back pain and comorbid depression exhibit higher levels of plasma IL-6 levels compared to healthy controls (Kim et al., 2012). In addition, chronic lower back pain patients have higher serum levels of TNFα and although the presence of comorbid depression did not influence TNFα levels, it did affect the therapeutic outcome to multidisciplinary treatment (Wang et al., 2010a).

A balance between pro- and anti-inflammatory activity is crucial to restrain excessive inflammation. An imbalance in the IL-6/IL-10 ratio is reported in depression, and significantly lower IL-10 levels are observed in chronic pain patients (Dhabhar et al., 2009, Steiner et al., 2008). Neopterin (a marker of IFNγ activity) is associated with hyperalgesia in depression and somatisation (Maes and Rief, 2012). Similarly, increased levels of the soluble IL-2 receptor are related to the somatic (pain and gastrointestinal symptoms) but not emotional aspects of depression (Euteneuer et al., 2012).

Fibromyalgia and rheumatoid arthritis are two chronic pain conditions with a significant inflammatory component that are highly comorbid with depression. Fibromyalgia patients have increased plasma and cerebrospinal fluid levels of pro- and anti-inflammatory cytokines and chemokines (Wang et al., 2008a, Wang et al., 2009a, Macedo et al., 2007, Kadetoff et al., 2012). In female fibromyalgia patients, depressive symptoms correlated with elevated C-reactive protein levels, a biomarker of inflammation (Menzies et al., 2011). Interestingly, stress in fibromyalgia was positively correlated with pain and depression and negatively correlated with plasma chemokine
CC-motif ligand 2 (CCL2) and IL-1β levels (Menzies et al., 2011). Rheumatoid arthritis (RA) is a chronic systemic autoimmune disorder, associated with significant pain and disability. RA patients often experience comorbid depression (Frank et al., 1988), and pain is positively correlated with depressive symptoms (Hawley and Wolfe, 1988). In RA, depression correlates with markers of inflammation such as IL-1β, IL-12, IL-18, TNFα and C-reactive protein (Low et al., 2009, El-Tantawy et al., 2008, Kojima et al., 2009, Lee et al., 2009). There is greater T-cell activation among RA patients who are depressed, with higher levels of IL-6 in patients under life stress (Zautra et al., 2004). These studies show that increased inflammation in chronic pain conditions is associated with depressed mood, an effect that is exacerbated by stress.

Due to the heterogeneity of depressive and chronic pain disorders, the fact that inflammatory markers vary dynamically over time, and the confounding factors such as treatment or disease stage, it is difficult to achieve a clear consensus on what exact glial cells and cytokines are altered in these conditions, and where. Nonetheless, an overall trend towards a shift in the balance to a proinflammatory cytokine profile in depression and chronic pain is observed. However, it should be noted that data on neuroimmune alterations in depression/pain are lacking. Thus, preclinical models are essential in order to examine the neurobiological mechanisms underpinning mood and pain disorders. A range of preclinical models of depression and persistent pain demonstrate disruptions in glial markers in prefrontal and limbic brain regions.

1.4.6.2 Preclinical evidence for neuroimmune alterations in depression

A multitude of evidence exists reporting glial changes in preclinical models of affective disorders. An elegant study reported that pharmacological inhibition of prefrontal glial metabolism seems to be sufficient to produce depressive- and anxiety-like behaviours in naïve rodents, similar to the effects of chronic stress (Banasr and Duman, 2008), while injection of the
gliotoxin flurocitrate into the hippocampus prevented the antidepressant effect of imipramine in the learned helplessness model of depression (Iwata et al., 2011). Moreover, brain IL-1 is essential for the development of chronic stress-induced depressive behaviours (Goshen et al., 2008). Similar to the clinical picture, reduced GFAP mRNA and protein in the hippocampus is reported following chronic unpredictable stress (Liu et al., 2011a, Liu et al., 2009) and early life stress has been shown to reduce expression of astrocytic markers (S100β, GFAP) in the anterior cingulate and precentral medial cortices in adulthood (Musholt et al., 2009). In contrast, maternal deprivation results increased astrocyte density in the hippocampus and cerebellum of neonatal and adolescent rats (Llorente et al., 2009, Marco et al., 2012, Lopez-Gallardo et al., 2012). Thus, early life stress may result in increased astrocyte activation during the post-stress period, with atrophy in later life. WKY rats exhibit increased astrocyte expression in the dorsal raphe nuclei (Pearson et al., 2006), but reductions in GFAP density in the PFC, ACC and amygdala (Gosselin et al., 2009). Reactive astrocytes with downregulated glial glutamate transporter in the hippocampus and increased circulating cytokines have been reported in FSL animals (Gomez-Galan et al., 2013, Carboni et al., 2010). Therefore, models of depression exhibit region-specific alterations in astrocyte expression in stress-sensitive areas and sites implicated in pain processing. Microglial activation has been reported following chronic stress in stress-sensitive brain regions such as the infralimbic, cingulate and orbitofrontal cortices, medial PFC, PAG, amygdala and hippocampus (Farooq et al., 2012, Tynan et al., 2010). Social defeat causes microglial activation in the amygdala, PFC, and hippocampus, with increased levels of IL-1β in microglia (Wohleb et al., 2011). Furthermore, chronic restraint stress results in microglial activation in the PFC (Hinwood et al., 2012). In addition, increased protein levels of proinflammatory cytokines in the PFC, hippocampus, and hypothalamus are reported following chronic stress (Tagliari et al., 2010, Mormede et al., 2002, Grippo et al., 2005, Garate et al., 2012) and olfactory bulbectomy (Borre et al., 2012b, Myint et al., 2007). Activation of the immune system by viral or bacterial mimetics induces depressive-like
behaviour which is associated with an increase in the expression of inflammatory cytokines in the brain (Gibney et al., 2013, Lawson et al., 2013). Therefore, alterations in immune markers are apparent across various models of depressive disorders, supporting clinical data for a role of neuroinflammation in the pathophysiology of depression.

**1.4.6.3. Preclinical evidence for enhanced inflammation in chronic pain**

While ample evidence supports a role for glia in the spinal cord in mediating nociceptive processing (for review see Vallejo et al., 2010, Bradesi, 2010), there is now increasing data highlighting an additional role for supraspinal immune processes and cytokines in pain processing. Intracerebroventricular injection of the proinflammatory cytokines IL-1β, IL-6, and TNFα induced thermal hyperalgesia in rodents (Oka et al., 1995, Oka et al., 1994, Oka et al., 1993, Oka et al., 1996). IL-1β injection to the medial preoptic area causes hyperalgesia, while microinjection into the ventromedial hypothalamus causes analgesia (Oka et al., 1995), indicating region-specific roles for these cytokines in nociceptive behavioural outputs. Peripheral noxious inflammatory stimuli activate supraspinal glial cells. For instance, formalin injection to the hind-paw results in microglial activation in the brainstem (Fu et al., 1999), while blocking glial activation reduces formalin-induced nociceptive behaviour (Lan et al., 2007). Microglia, both spinally and supraspinally, are involved in the development of neuropathic pain, while in comparison, astrocytes have been implicated in the maintenance of this pain state (Romero-Sandoval et al., 2008, Colburn et al., 1997, Wei et al., 2008, Raghavendra et al., 2003). Persistent pain in rodents leads to glial activation, cytokine and chemokine release in areas remote from the site of injury. Across various models of peripheral nerve injury, increases in TNFα, IL-1β, IL-6 and NF-κB are reported in whole brain homogenates (Jia et al., 2010, Liu et al., 2007, Xie et al., 2006), and discrete regions including the hippocampus, brainstem, cingulum, striatum, locus coeruleus and PFC (Zhu et al., 2009, Wan et al., 2007, Apkarian et al., 2006, Al-Amin et al., 2011, Ignatowski et al., 1999, Covey et al., 2002). CCI results in early microglial activation and sustained astrocyte reaction in the RVM, PAG and hypothalamus (Takeda et al., 2009b, Wei et al., 2008). The ACC is a key
region for modulation of nociceptive behaviour, and studies have shown that SNL and complete Freund’s adjuvant are associated with increased activation of astrocytes and TNFα in the ACC (Kuzumaki et al., 2007, Jia et al., 2007) and propentofylline, a glial modulator, injected directly into the cingulate cortex 24h before ligation had anti-allodynic and anti-hyperalgesic effects (Kuzumaki et al., 2007). Finally, NF-κB expression was increased in the hippocampus and ACC but was downregulated in the somatosensory cortex and amygdala following CCI (Chou et al., 2011b, Chou et al., 2011a), indicating differential pain-related expression depending on the region examined, effects which may have implications for descending control of pain and affective pain behaviour. Taken together, these data suggest that region-specific glial activation and enhancement of pro-inflammatory cytokines plays a role in the central processing of pain following peripheral nerve injury and inflammatory insult. Supraspinal glial modifications are also implicated in the affective processing of chronic pain. Persistent pain leads to activation of astrocytes in the ACC, an effect related to affective pain behaviour (Chen et al., 2012a), and increased TNFα expression in the amygdala of complete Freund’s adjuvant-treated animals was associated with anxiety-like behaviour (Chen et al., 2013). Astrocyte activation occurs in the cingulate and orbitofrontal cortices in models of peripheral nerve injury, which correlates with anxiety-like behaviours (Kuzumaki et al., 2007, Narita et al., 2006b, Fuccio et al., 2009). The formalin-induced conditioned place aversion model, which induces a negative affective state caused by inflammatory nociceptive stimuli, resulted in an increase in astrocyte activation and proinflammatory cytokines in the ACC (Lu et al., 2011). Therefore, increased pain-induced glial activation in brain regions responsible for the modulation of nociception and affect is associated with increased anxiety-like behaviour.

1.4.6.4 Inflammation in the interaction between depression and pain – preclinical evidence

As discussed in Section 1.3, inflammatory nociceptive responding is enhanced in almost all models of depression (Burke et al., 2010, Shi et al., 2010b, Wang et al., 2012a, Wang et al., 2010b, Gameiro et al., 2005, Bardin
et al., 2009, Andre et al., 2005, Rivat et al., 2010, Kim et al., 2012, Uhelski and Fuchs, 2010), indicating that altered immune processing may underlie the hyperalgesia observed in these models. In the neonatal limited bedding (NLB) model of early life stress, prostaglandin-induced mechanical hyperalgesia is prolonged compared to non-stressed controls (Green et al., 2011). Moreover, sound-stress applied in adulthood exacerbated mechanical hyperalgesia in NLB rats, an effect prevented by spinal blockade of IL-6 receptor gp130 or TNF-receptor 1 (Alvarez et al., 2013). Repeated social defeat has been shown to increase gene expression of iNOS and COX-2 in the spinal cord, an effect associated with decreased mechanical withdrawal thresholds (Rivat et al., 2010) and inhibiting microglial p38 in the spinal cord prevents water-avoidance stress-induced visceral hyperalgesia (Bradesi et al., 2009). As such, stress-induced spinal inflammation is associated with enhanced mechanical and visceral hyperalgesia. The co-occurrence of pain and depression results in enhanced neuroinflammation in supraspinal brain regions. Reserpine-induced pain-depression syndrome is associated with increased TNFα and IL-1β in the cortex and hippocampus of rats and mice (Arora et al., 2011, Xu et al., 2013). Depressive-like behaviour following complete Freund’s adjuvant was associated with increased IL-6 mRNA in the hippocampus (Kim et al., 2012). Furthermore, spared nerve injury in mice resulted in mechanical allodynia and depressive-like behaviour, an effect accompanied by increased IL-1β mRNA in the frontal cortex and increased GFAP mRNA in the PAG (Norman et al., 2010b). Chronic restraint stress in these animals resulted in exacerbated mechanical allodynia and depressive-like behaviour, and a further enhancement of IL-1β mRNA in the frontal cortex (Norman et al., 2010b). The same authors reported that socially isolated mice with nerve injury also exhibited depressive-like behaviour with increased IL-1β mRNA in the frontal cortex (Norman et al., 2010a). Furthermore, IL-1β mRNA was increased 10 days following spared nerve injury in the brainstem and PFC of the stress-sensitive WKY rat, and IL-1β levels in the ipsilateral PFC correlated with mechanical sensitivity in this model (Apkarian et al., 2006). Thus, the current studies indicate that the combination of depression and pain is associated with enhanced
inflammation in the central nervous system. The behavioural output may represent molecular, cellular, chemical, structural and functional alterations in the brain triggered by neuroinflammatory changes in specific brain areas responsible for parallel processing of emotion and nociception.
1.4 Convergence and interactions of neural substrates implicated in depression and pain

As discussed above, a number of possible substrates, mediators and pathways modulate emotional and nociceptive processing, any or all of which may drive somatic and psychological symptoms observed in pain and depression.

Presented in Fig. 1.5 is a schematic depicting the possible interaction between these substrates and how alterations in these systems relate to each other and may account for the behavioural and somatic changes observed in depression and chronic pain disorders. In brief, chronic stress, injury and pain may cause activation of microglial cells and hyperdrive of the HPA...
Chapter 1 – Introduction

axis with damage to the hippocampus, reduced BDNF and neurogenesis, and a loss of the anti-inflammatory effects of glucocorticoids. This may contribute to an imbalance between pro- and anti-inflammatory processes leading to unrestrained inflammatory activity which has a detrimental effect on neurons involved in emotional and nociceptive processes. Multiple astrocytic functions may be altered due to excessive exposure to cytokines and reactive oxygen/nitrogen species, ultimately leading to downregulation of glutamate transporters, impaired glutamate reuptake, and increased glutamate release, contributing to neurodegeneration. Moreover, inflammation induces the enzyme indoleamine 2,3 dioxygenase (IDO) which shunts tryptophan down the kynurenic acid pathway resulting in reduced serotonin availability and formation of quinolinic acid, a potent NMDA agonist and stimulator of glutamate release, enhanced levels of which are neurotoxic. Resultant decrease in serotonin availability and degeneration of monoaminergic neurons reduces the availability of serotonin and noradrenaline in brain regions that regulate emotion and nociception, which may enhance or provoke pain and depression. Compromised function of cortical-limbic circuitry in depression and chronic pain may further disrupt autonomic, neuroendocrine and neuroimmune regulation. Antidepressant drugs have been shown to act on the monoaminergic system (Artigas et al., 2002), have anti-inflammatory characteristics (Bianchi and Panerai, 1996), alter HPA axis functionality (Mason and Pariante, 2006), and enhance neurotrophins (Castren, 2004), effects which may act alone or in unison to attenuate pain and depression simultaneously. Although the link between depression and pain is clear, further research is required in order to elucidate the underlying mechanisms associated with this disabling comorbidity.
1.6 Overall objectives and experimental approach

The main objective of the work presented herein was to improve our understanding of the effect of an affective phenotype on nociceptive responding in animal models. As evidence by the above review of the literature, a range of animal models exhibit altered nociceptive responding in various tests of acute and chronic pain. The aim of this thesis was to characterise nociceptive responding in two rat models of affective disorders, prior to and following peripheral nerve injury. A further aim of this thesis was to examine the underlying mechanisms that may mediate the relationship between depression, early life stress and pain.

Hypothesis

Nociceptive and neuropathic-pain related behaviour would be enhanced in two rat models of affective disorders, an effect that would be accompanied by altered monoamine levels and/or neuroimmune mediator gene expression in the spinal cord and brain regions responsible for processing emotion and pain. Moreover, behavioural and neurobiological parameters would be differentially altered by monoaminergic and anti-inflammatory treatments in the presence or absence of a depressive-like phenotype.

The specific objectives of this thesis were:

(1) To examine nociceptive responding to thermal and mechanical stimuli prior to and following the induction of neuropathic pain, in an animal model of depression, the olfactory bulbectomised (OB) rat. The OB rat is a well-validated and reproducible model of depression (for review see Kelly et al., 1997, Song and Leonard, 2005), which results in a permanently altered state that can be used to model chronic depression (Willner and Mitchell, 2002). Removal of the olfactory bulbs induces behavioural, neurotransmitter, neuroendocrine and immune changes resembling those reported in depressed patients (for review see Kelly et al., 1997, Song and Leonard, 2005). The first aim was to confirm development of OB-depressive-like syndrome in the model using the open field test. Following this, nociceptive
responding to mechanical (von Frey) and thermal stimuli (acetone drop test and Hargreaves test) was assessed. Previous data from our laboratory have demonstrated altered mechanical, but not thermal, nociceptive responding in this model (Burke et al., 2010). We aimed to confirm and elaborate on these findings. Despite the fact that neuropathic pain is highly comorbid with depression and particularly difficult to treat (Radat et al., 2013), relatively few studies have examined if neuropathic pain responding is altered in the presence of a depressive-like phenotype. Thus, the work presented herein sought to examine the development of neuropathic pain-related behaviour (using the spinal nerve ligation model) in response to mechanical and thermal stimuli in the OB model (Chapter 3).

(2) An in-depth investigation into all of the potential mechanisms implicated in depression and pain is evidently beyond the scope of this dissertation. Therefore, the experiments presented herein investigated two of these systems, namely the monoaminergic system which has a well-known role in depression and pain co-morbidity, and neuroinflammation, which is less well studied but provides a promising therapeutic target. In order to evaluate the possible role of monoamine and neuroimmune mediators in the behavioural responses in the OB model (depression, nociception and the interaction between these processes), levels of monoamines and the mRNA expression of cytokines and markers of microglial and astrocyte activation were examined in key brain regions responsible for regulating emotion and pain – the prefrontal cortex, amygdala, thalamus and hippocampus (Chapter 3).

(3) As the monoaminergic antidepressant amitriptyline (AMI) is one of the most widely prescribed treatments for neuropathic pain, a further aim of this thesis was to evaluate the effect of chronic treatment of this pharmacological agent on nociceptive responding, prior to and following nerve injury, in the presence and absence of a depressive phenotype. The antidepressant-like activity of AMI is confirmed by reversal of the OB-induced hyperactivity in the open field test, a gold standard for assessing
antidepressant activity in the model. Subsequent to this, nociceptive responding to mechanical and thermal stimuli was evaluated prior to and following SNL, in both sham and OB animals. Alterations in monoamines and immune mediators in discrete brain regions were then evaluated to examine possible mechanisms of action of the drug that would underlie the behavioural alterations observed (Chapter 4).

(4) Neuroimmune process, particularly microglia, are now well-recognised to play a role in the development of chronic pain and depressive-like states, however relatively few studies have examined the role of immune mediators in the interaction between depression and pain. Thus, a further aim of this thesis was to determine the effect of chronic and acute minocycline (a microglial inhibitor) treatment on depressive-like behaviour, nociceptive and neuropathic pain responding in the OB rat. Following on from this, alterations in immune mediators in the spinal cord and discrete brain regions were then evaluated to examine possible mechanisms of action of chronic minocycline treatment that may underpin the behavioural alterations observed (Chapter 5).

(5) Early life stress is known to increase the risk of development chronic pain conditions and psychiatric disorders in adulthood (Gutman and Nemeroff, 2003, Nemeroff, 2004b, Fillingim and Edwards, 2005, Davis et al., 2005). The maternal deprivation (MD) model used in Chapter 6 involves a single prolonged (24h) period of separation of mother and pups on postnatal day 9 (Ellenbroek et al., 1998). This insult, which contains sensorimotor, nutritional and temperature components, provokes behavioural, neurochemical and immune changes in adulthood that resemble those seen in psychiatric disorders such as depression, anxiety and schizophrenia (Marco et al., 2009, Viveros et al., 2009). This study examined nociceptive responding prior to and following peripheral nerve injury in the MD model and examined if MD-induced alterations in behavioural responding were associated with concomitant alterations at the neuroimmune level by evaluating mRNA expression of markers of glial activation and cytokines in the prefrontal cortex and hippocampus.
Chapter 2

General Methods
2.1 Animal husbandry

Male Sprague Dawley rats (Charles River, Margate, UK) were used for the majority of studies (apart from studies described in Chapter 6). Sprague Dawley rats weighed 175-220g on arrival and were housed singly in plastic-bottomed cages (45 × 25 × 20 cm) containing wood shavings as bedding (changed weekly), in a temperature controlled room (20 ± 2°C), relative humidity of 40-60%, with a 12:12h light-dark cycle (lights on at 0700h). Rats were fed a standard laboratory diet of rat chow pellets (2014 14% rodent diet, Harlan Laboratories UK Ltd., Leics, UK); food and water were available ad libitum. The experimental protocol was carried out in accordance with the guidelines and approval of the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609 and every effort was made to minimise the number of animals used and their suffering.

2.2 Olfactory bulbectomy (OB) model of depression

Bilateral olfactory bulbectomy (OB) was performed as outlined previously (Roche et al., 2007, Burke et al., 2010). Rats were anaesthetised with isoflurane (Abbot Laboratories, UK, 5% induction, 2% maintenance in 0.5L/min O₂), the head shaved, and placed in ear bars in a stereotaxic frame (Harvard Apparatus, MA, USA). Fucidic acid 1% viscous eye drops (Fucithalmic®, LEO Laboratories Ltd., Dublin) were applied to each eye to prevent drying out during surgery, and the head was cleaned with alcohol swabs and betadine (7.5% w/v, iodinated povidone, Videne®, Ecolab Ltd., Leeds, UK). After the application of local anaesthetic (bupivacaine HCl 0.25%), a midline sagittal incision was made in the skin overlying the skull using a sterile scalpel blade. The periosteum was cleared from the skull, cleaned using sterile saline and cotton buds, and using 4 bulldog clips the incision was held open such that bregma and the frontal sutures were clearly visible. Two burr holes of 2mm diameter were drilled into the skull 5mm
rostral to bregma and 2mm lateral to the midline using a sterilised drill bit (no. 8). The olfactory bulbs were removed by gentle aspiration with a blunt hypodermic needle (15 gauge) attached to a venturi vacuum pump and care was taken not to damage the frontal cortex. The burr holes were then plugged with a haemostatic sponge (Septodont, supplied by Lohans Pharmacy, Galway, Ireland) to control bleeding. Sham-operated animals were treated in the same manner but the bulbs were left intact. Antiseptic dusting powder (CX chlorhexidine powder, Ecolab, Ireland) was applied to the skull and the wound was closed with sterile wound clips (7.5mm x 1.75mm, Aesculap AG & Co. KG, Tullingen, Germany). Betadine was applied to the wound once closed. Sterile saline (1ml) was administered intraperitoneally (i.p.) to promote rehydration and the animals were placed in a clean cage with fresh bedding in a warm environment until fully recovered from anaesthesia. Animals were handled daily following surgery and lesions were verified by gross inspection on completion of the study. Animals were eliminated from the analysis if the bulbs were not completely removed or if damage extended to the frontal cortex. Sham-operated animals were removed from the analysis if there was any damage to the bulbs or the frontal cortex.

![Fig. 2.1](image_url) Rat brain with location of the olfactory bulbs (OB). Adapted from (Neville and Haberly, 2004)


2.3 L5-L6 Spinal nerve ligation (SNL) model of neuropathic pain

Spinal nerve ligation (SNL) is a well-characterised model of chronic neuropathic pain and was carried out as previously described (Moriarty et al., 2012, Kim and Chung, 1992). Unilateral ligation of the L5-L6 segmental spinal nerves results in long-lasting and robust mechanical and cold allodynia, and thermal hyperalgesia of the ipsilateral hind-paw when compared to the contralateral hind-paw and non-ligated counterparts.

The rats were anaesthetised with isoflurane (Isoflo®, Abbott Laboratories, Berkshire, UK, 5% for induction and 2.5% for maintenance in 0.6 L/min O₂). Fucidic acid viscous eye drops were applied to each eye to prevent drying out during surgery. The rats were anaesthetised with isoflurane (Isoflo®, Abbott Laboratories, Berkshire, UK, 5% for induction and 2.5% for maintenance in 0.6 L/min O₂). Fucidic acid viscous eye drops were applied to each eye to prevent drying out during surgery. The fur lateral to the midline on the left-hand side at the lower lumbar and sacral regions was clipped. The incision area was swabbed with an alcohol swab (UHS, Enfield, UK) and scrubbed with betadine. 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 clips (Fine Science Tools, cat. No 18050-28, supplied by InterFocus Ltd., Cambridge, UK) were used to hold the skin back and a retractor (Fine Science Tools, cat. No 17005-04) was used to hold open the surgical cavity. A tear-drop shaped piece of muscle was excised with a scalpel blade and scissors, and paraspinal muscles were removed using a toothed forceps to visualise the L6 transverse process. The transverse process was then removed using a small rongeurs (Fine Science Tools, cat no. 16021-14), exposing the L4 and L5 spinal nerves. 6-0 silk suture (0.07mm diameter, Deknatel®, supplied by InterFocus Ltd., Cambridge, UK) was looped around the L5 nerve using a fine curved forceps (Fine Science Tools, cat no. 11274-20) and the nerve was tightly ligated using two knots. The L6 nerve is located under the sacrum and was isolated using a small hook (Fine Science Tools, cat. No. 1006-12) and was also ligated. Care was taken to avoid damage to the L4 spinal nerve which contains motor efferents to the hind-paw. Sham-operated (NSNL) rats were treated in the same manner however the L5 and L6 nerves were exposed but not ligated with 6-0 silk suture. Haemostasis was confirmed before closing the wound using an internal suture for the muscle layer (Vicryl Rapide 4-0,
Ethicon, Johnson & Johnson, NJ, USA) and the skin layer was closed with sterile wound clips. Rats were injected i.p. with 1 ml of sterile saline to counter dehydration. Animals were placed in a clean cage with fresh bedding in a warm environment until fully recovered from anaesthesia. Animal health was closely monitored in the 24 hours post-surgery and each day thereafter.

**Fig. 2.2** Schematic diagram showing the dorsal view of the bony structures at the lower lumbar and sacral levels. Reproduced from (Chung et al., 2004). (A) Location of L5 and L6 spinal nerves. (B) Bony structures after removal of paraspinal muscles. (C) Left side after removal of the L6 transverse process and fascia.
2.4 Behavioural Testing

All testing was carried out by an experimenter blind to group identity, and the arenas were thoroughly cleaned with a mild disinfectant solution (Milton and warm water, 1:10) between each testing session.

2.4.1 Behavioural measurements of locomotor activity and affective behaviour

2.4.1.1 Open Field Test

On the experimental day, each animal was removed from the home cage during the light phase, placed in a fresh cage and brought to a separate procedure room. Animals were placed singly into a brightly lit (lux 200-400) novel open field arena (diameter 75cm) with a white floor (plastic-covered wood flooring) and reflective walls (Height 50cm, aluminium). A camera was placed above the arena and the test was recorded to DVD for subsequent analysis. Locomotor activity (distance moved, cm) and time spent in and latency to enter the inner zone (diameter 50cm) was assessed using a computerised video tracking system (EthoVision®, Versions 3.1, and 8 Noldus, Wageningen, Netherlands, see appendix C) over a 5 minute period. Manually rated behaviours (time spent rearing and grooming) were rated using EthoVision. Faecal pellet count was measured as an indicator of emotionality in OB rats (Walsh and Cummins, 1976) and as a general measure of anxiogenic-like behaviour.
Fig. 2.3 Open field arena (75cm) showing inner zone (50cm).
2.4.2 Behavioural measurements of nociceptive responding

Nociceptive responding assessed prior to SNL was expressed as an average response value for the left and right hind-paw, whereas following SNL surgery nociceptive response values for the ipsilateral and contralateral hind-paws are reported separately.

2.4.2.1 von Frey test for mechanical withdrawal thresholds

The arena used for von Frey testing consisted of a six-compartment Perspex arena (11cm × 20cm × 15cm), with a Perspex lid with air-holes, which allowed rats to move freely within. The arena was placed on a raised wire mesh flooring to allow the experimenter to access the hind-paw from below. Rats were habituated to the arena for 20 minutes prior to testing. von Frey filaments (Touch-Test® Sensory Evaluators, North Coast Medical, Inc., Gilroy, CA, USA) of different forces (0.07g – 100g) were used to determine the lowest filament force to elicit a response (Tal and Bennett, 1994) and 50% withdrawal thresholds (Chaplan et al., 1994). Starting with the 2g filament, filaments were applied perpendicular to the plantar surface of the hind-paw, aiming for the area between the 3rd and 4th toes (the area which is most sensitive following SNL Chung et al., 2004), with sufficient force to cause slight buckling of the filament, for up to a maximum of 5 seconds or until flinching, licking or withdrawal of the paw occurred. Filaments were applied to both right and left hind-paws five times (alternating between paws). First, the 2g filament was applied to the right hind-paw of all animals, and then applied to the left hind-paw of each animal. If one or more positive responses were recorded to application of the 2g filament, lower force filaments were applied (to the right hind-paw of all rats, then the left, as above) in descending order until no positive responses were observed. Next, filaments of increasing force were applied until a 100% positive response (5 positive responses to 5 applications) was observed for two consecutive filaments. The percentage response to each filament was calculated using the following formula: ([no. of responses/5] × 100). The filament force eliciting a 50% response was calculated by plotting a non-
linear regression curve of the percentage response versus filament force for each rat. For Chapters 3-5, testing was carried out in procedure rooms where the lux was approximately 30 (same as the holding room). For Chapter 6, as rats were on a reverse light-dark cycle, testing was carried out under red light conditions however a low white light illumination was provided (lux = 5) in order for the experimenter to visualise the paw responses to filament application.

Figure 2.4: (A) Schematic illustration of the apparatus used for von Frey testing. (B) Photograph of von Frey testing procedure. (C) Plantar surface of rat hind-paw showing the area (shaded portion) at which the von Frey filament was applied.
2.4.2.2 Hargreaves Test for thermal nociceptive responding

The Hargreaves apparatus (Plantar Analgesia Meter, IITC Lifesciences Inc., Woodland Hills, CA, USA) was used to measure thermal nociception and assesses heat hyperalgesia, as previously described (Hargreaves et al., 1988). The apparatus consisted of a six-chambered Perspex arena placed on top of a raised glass panel heated to 30°C. Rats were placed in individual chambers (15 × 20 × 11cm) and allowed to habituate for 20 minutes. A moveable radiant heat source (light) was positioned underneath the glass and a guide light (idle intensity of 1%) was used to focus the beam on the plantar surface of the hind-paw. A focused beam of radiant light (active intensity of 30%, which corresponds to 53 degrees celsius, Torres et al., 2007) was used to heat the plantar surface of the hind-paw (between the 3rd and 4th toes) and the latency to flinch, lick or withdraw the hind-paw was recorded. To prevent tissue damage, a cut-off parameter of 20 seconds was set. If no response occurred during this time, the cut-off time of 20 seconds was recorded as the latency. Each animal received 8 trials in total, four per paw alternating between left and right, with the left paw of each animal tested first, then the right paw on each animal, and so on. A 3 minute interval between testing the other paw was observed. The average withdrawal latency of the last three trials was calculated for each hind-paw.

Fig. 2.5 Hargreaves apparatus used for examining thermal nociceptive responding. Picture: Douglas Mental Health University Institute, 2013
2.4.2.3 Acetone drop test for responding to a cold innocuous stimulus

The apparatus used for the acetone drop test was the same as that used for the von Frey testing. The protocol was similar to that of Choi et al. (1994). Rats were habituated to the arena for 20 minutes prior to testing, or when von Frey and acetone drop testing were carried out on the same day, a 20 minute interval was observed between both tests, with von Frey testing first followed by the acetone drop test. Polyethylene tubing (2mm internal diameter, 3mm external diameter, Portex® Fisher Scientific, Dublin, Ireland) attached to a 1ml syringe was used to apply acetone (100%, 650501: Sigma-Aldrich, Dublin, Ireland). The drop of acetone (20 μl) was applied to the plantar surface of the hind-paw (between the 3rd and 4th toes) without mechanically stimulating the hind-paw with the tubing. As acetone evaporates, it causes a cooling sensation, which is normally innocuous, but results in reduced paw withdrawal latency and increased frequency of responding following peripheral nerve injury, indicative of cold allodynia. Latency to first response (using a stop-watch) and withdrawal frequency within 60 seconds was recorded for each trial. A positive response was considered as a flinch, lick or withdrawal of the hind-paw. If the animal did not respond within 60 seconds, this value was taken as the latency. Each animal received 8 trials in total, four per paw, alternating between left and right (as above), with at least a 3 minute interval between testing the other paw. The average of the 4 trials was calculated for each hind-paw.

2.4.2.4 Place escape-avoidance paradigm for affective pain behaviour

The place escape avoidance paradigm (PEAP) attempts to differentiate between the affective and somatic components of pain in models of unilateral pain (La Buda and Fuchs, 2000). Essentially, this paradigm creates a conflict between the aversive light compartment of a two compartment arena and aversive noxious mechanical stimulation in the dark compartment, thus giving a choice to move to the light side in order to escape and/or avoid noxious stimulation in the dark side. When the animal is located within the dark side of the chamber, the ipsilateral hind-paw is stimulated, and when the animal is within the light side (lux 200), the
contralateral hind-paw is stimulated. The expectation is that rats will move into the harsher, brighter confines of the light side to avoid stimulation of the allodynic paw in the dark side. The arena (30cm x 30cm x 30cm Perspex box which comprised of a transparent light half, and a dark half, with a narrow opening, allowing unrestricted movement) was placed on an elevated mesh floor. The test was recorded from a camera placed above the light-side chamber, and time spent in the light chamber was assessed using a computerised video tracking system (EthoVision®) over a 30 minute period. The rat was placed in the light-side chamber (without habituation to the arena) and a 60g von Frey filament was applied to the plantar surface of the hind-paw (between the 3rd and 4th toes), every 15 seconds, to the ipsilateral hind-paw while in the dark side, and contralateral hind-paw while in the light. The rat was considered to be in a particular side of the arena when its head and two front paws were in that side.

Fig. 2.6 Schematic depicting the apparatus used for the place escape/avoidance paradigm. Noxious stimulation is applied to the contralateral (uninjured) hind-paw when in the light chamber and the ipsilateral (injured) hind-paw when in the dark chamber. The expectation is that the animal will avoid the noxious stimulation in the dark and move towards the aversive light chamber.
2.5 Animal sacrifice and tissue collection

Rapid decapitation was used as the humane experimental endpoint for all studies.

2.5.1 Blood collection

Following decapitation, trunk blood was immediately collected in 15ml plastic test-tubes, allowed to clot (for collection of serum) and stored at 4°C until centrifugation one hour later, at which point the serum was removed and stored at -80°C until analysis of corticosterone levels.

2.5.2 Removal of brains

Following decapitation, an incision was made using a scissors along the top of the head and the skin pulled back to expose the skull. The optic ridge between the eyes and the back of the skull was broken with a rongeur. Using a scissors, a cut was made carefully along the midline of the skull from the back, maintaining pressure away from the brain surface, and the parietal and frontal skull was removed. The remaining bone along the sinus between the olfactory bulbs and frontal cortex was carefully removed, as was the bone over the nasal cavity and eye socket. The dura mater was removed, the trigeminal nerve was cut and the brain removed from the skull using a curved forceps.

2.5.3 Brain dissection

Immediately after decapitation, discrete brain regions (prefrontal cortex, hippocampus, thalamus, and amygdala) were dissected on an ice-cold plate which was sprayed with RNaseZap® (Invitrogen, Dublin, Ireland) to remove RNases. Each region was divided into right and left, placed into RNase free microfuge tubes and snap-frozen on dry ice and stored at -80°C for further analysis.

2.5.4 Removal of spinal cords

After decapitation, an incision was made down the length of the back of the carcass. The muscle was removed using a toothed forceps until the spinal column was visible. A small ronguer was used to make two incisions in the
vertebra at the proximal end of the column, lateral to the cord, such that the dorsal part of the bone could be removed. The thoracic and lumbar vertebrae were removed with rongeurs, the cord was removed from the spinal cord by carefully lifting it with a forceps and cutting the nerve endings. The spinal cord was then post-fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 24 hours then stored in 20% sucrose with 0.1% sodium azide until processing. For chapter 3, laminectomy was performed as described above, as it is considered preferable for immunohistochemical studies. For chapter 5, the spinal cord was flushed from the column using a syringe filled with ice-cold sterile PBS as this is sufficient for PCR studies. The lumbar enlargement containing the L4-L6 region was isolated, and the tissue was snap-frozen on tin-foil and stored at -80°C until further analysis by qRT-PCR.

2.6 Analysis of inflammatory mediator gene expression using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.6.1 RNA isolation

Total RNA was extracted from homogenized brain or spinal cord tissue (<30mg) using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Düren, Germany). This method involved homogenising tissue (Polytron tissue disrupter, Ultra-Turrax, IKA®-Werke, Staufen, Germany) in 354µl of RA1 lysis buffer containing 1% β-mercaptoethanol (Sigma, Dublin, Ireland). Homogenates were filtered in a Nucleospin filter, centrifuged at 14,000g for 1min and the lysates treated with 350µl of 70% molecular grade ethanol (Sigma, Dublin, Ireland) and mixed by pipetting up and down 10 times. The samples were transferred to Nucleospin RNA II columns and centrifuged at 14,000g for 30 seconds to bind the RNA. After desalting the column membrane with 350µl of membrane desalting buffer (MDB, supplied with kit), columns were spun at 14,000g for 1 minute. DNA was removed using rDNase and DNase reaction buffer (supplied). rDNase was diluted 1:10 in DNase reaction buffer and 95µl of this solution
was pipetted directly onto the centre of each column and allowed to stand for 15 minutes at room temperature. The rDNase was then inactivated by the addition of 200µl RA2 buffer, and the columns were then centrifuged at 14,000g for 30 seconds, then 600µl of RA3 wash buffer was added to the column followed by centrifugation at 14,000g for 30 seconds. The eluent was discarded and 250µl of RA3 wash buffer was added followed by centrifugation at 14,000g for 2 minutes. The columns were then placed in RNase-free collection tubes and the RNA was eluted by the addition of 60µl of RNase-free water (Sigma, Dublin, Ireland) followed by centrifugation at 14,000g for 1 minute. The eluted RNA was then stored at -80°C until quantification and reverse transcription.

2.6.2 RNA quantification and equalization

The quantity, purity and quality of RNA was assessed using a Biophotometer plus (Eppendorf, Stevenage, UK) or Nanodrop (ND-1000, Nanodrop, Labtech International, Ringmer, UK). RNA quantity was determined by measuring optical density (OD) at 260nm. RNA quality was determined by measuring the ratio OD\(_{260}/OD\(_{280}\) where a ratio of approximately 1.8-2.1 was deemed indicative of pure RNA. All RNA samples with a ratio >1.6 were accepted. Prior to cDNA synthesis, all samples were equalised to the same concentration of RNA (2-4µg/20µl) using RNase free water. Equalised samples were then stored at -80°C until reverse transcribed.

2.6.3 Reverse Transcription of RNA

A high capacity complementary DNA (cDNA) kit (Applied Biosystems, Warrington, UK) was used to reverse transcribe RNA samples. 10µl of equalised RNA was added to an equal volume of 2X master mix in a PCR mini-tube. The 2X master mix was prepared as follows: 2.0µl 10X RT buffer, 0.8µl 25X dNTP mix, 2.0µl 10X RT random primers, 1.0µl Multiscribe Reverse Transcriptase, 4.2µl RNase free water. A negative control was included using the master mix with an equal volume of RNase-free water. Samples were then placed in a Doppio thermal cycler and incubated at 25°C for 10 minutes, 37°C for 2 hours, 85°C for 5 minutes. A
final incubation maintained the cDNA at 4°C and the resultant cDNA was stored at -80°C until required for quantification by qRT-PCR.

2.6.4 Quantitative Real-time PCR

Gene expression of target proteins were determined using commercially available TaqMan gene expression assays (Applied Biosystems, Warrington, UK) containing specific forward and reverse target primers and FAM-labelled MGB probes, as previously described (Kerr et al., 2012, Kerr et al., 2013). β-actin gene expression was used as an endogenous control to normalise gene expression between samples and was quantified using a β-actin endogenous control assay containing specific primers and a VIC-labelled MGB probe. Assay IDs for the genes of interest examined were as follows:

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>Rn00709342_m1</td>
</tr>
<tr>
<td>CD40</td>
<td>Rn01423583_m1</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rn00566603_m1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Rn00580432_m1</td>
</tr>
<tr>
<td>TNFα</td>
<td>Rn99999017_m1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Rn00561420_m1</td>
</tr>
<tr>
<td>IL-10</td>
<td>Rn00563409_m1</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Rn00585674_s1</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Rn00573488_m1</td>
</tr>
<tr>
<td>β-actin (housekeeping gene)</td>
<td>Rn00667869_m1</td>
</tr>
</tbody>
</table>

Table. 2.1. Assay ID’s for TaqMan probes
A reaction mixture was prepared and stored on ice for each target gene. This consisted of 1.25µl target primers, 1.25µl β-Actin (multiplex version) and 12.5µl TaqMan master mix per sample. cDNA samples were diluted 1:4 and 10µl of each diluted sample was pipetted in duplicate onto a MicroAmp® optical 96 well plate. 15µl of the relevant reaction mixture was then added to each well giving a total reaction volume of 25µl. Negative controls containing the master mix without cDNA were included per probe. Plates were then covered with adhesive covers and spun at 1000g for 1 minute to ensure complete mixing. The plate was then placed in the real time PCR thermocycler (ABI Prism 7500 instrument, Applied Biosystems, Warrington, UK) pre-set to run the following Relative Quantification protocol: step 1: 95°C for 10 minutes, step 2: 95°C for 15 seconds followed by one minute at 60°C. Step 2 was repeated 40 times and the fluorescence read during the annealing and extension phase (60°C) for the duration of the programme.

2.7.5 Analysis of RT-PCR Results

Amplification plots and cycle threshold (Ct) values were examined using Applied Biosystems 7500 System SDS Software 1.3.1 and exported to Microsoft Excel for final analysis. The 2−ΔΔCt method was used to determine gene expression (Livak and Schmittgen, 2001). This method is used to assess relative gene expression by comparing gene expression of experimental samples to control samples, allowing determination of the fold change in mRNA expression between experimental groups. This method involves 3 steps: (1) Normalisation to endogenous control (β-actin) where ΔCt is determined: ΔCt = Ct Target gene - Ct Endogenous control; (2) Normalisation to control sample where ΔΔCt is determined: ΔΔCt = ΔCt Sample - average ΔCt of Control group; and (3) where the fold difference is given by 2−ΔΔCt. The 2−ΔΔCt values for each sample were then expressed as a percentage of the average of the 2−ΔΔCt values for the control group. In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined.
Fig 2.7 Example of amplification plots for (A) the endogenous control β-actin and (B) GFAP in the rat prefrontal cortex.
2.8 High-performance liquid chromatography coupled to electrochemical detection for the quantification of monoamine levels

Quantification of concentrations of monoamines by high-performance liquid chromatography (HPLC) was carried according to methods described previously (Burke et al., 2010, Seyfried et al., 1986). Brain regions were weighed, kept cold on ice and homogenised in 1ml of buffer containing mobile phase (see appendix A2) spiked with 2ng/20µl of internal standard, N-methyl-5-hydroxytryptamine (N-methyl-5HT). Homogenised tissue was then centrifuged at 14,000g for 15 minutes at 4°C. A 20µl sample of supernatant was injected into and analysed by a Shimadzu HPLC system (Mason Technology, Dublin) consisting of a CTO-6A oven, LC-10AT pump, a L-ECD 6A detector, the Sil-9A sample injector and a C-R5a chromatopac with a 5 micron pre-column and a reverse-phase analytical column (LiChrosorb RP- C18, length 250mm and internal diameter 4mm). Electrochemical detection (at 0.8V) was used to determine peak heights of monoamines and their metabolites. The temperature was maintained at 30°C and the flow rate through the system was 1ml/min. Peak heights for the standard mix of 2ng/20ul for noradrenaline (NA), 3,4-Dihydroxyphenylacetic Acid (DOPAC), Dopamine (DA), 5-Hydroxyindole-3-Acetic Acid (5HIAA) and Serotonin (5HT) were taken before the first sample, after every 10 samples, and after the last sample had been analysed.

The peak heights for each monoamine were obtained from the chromatographs and recorded for each sample and the standard mix. The mean of the standard mixes were then taken to determine the relative retention factor (RRF) for each neurotransmitter.

\[
\text{RRF} = \frac{(\text{CONC}_{\text{IS MIX}} \times \text{PH}_{\text{NT MIX}})}{(\text{CONC}_{\text{NT MIX}} \times \text{PH}_{\text{IS MIX}})}
\]

As the mix has the same constituents as the samples, one can assume that the RRF of the mix = the RRF of the sample, if analysed under the same conditions.

\[
\text{CONC}_{\text{NT}} = \frac{(\text{PH}_{\text{NTSAMPLE}} \times \text{CONC}_{\text{ISSAMPLE}})}{(\text{PH}_{\text{ISSAMPLE}} \times \text{RRF}_{\text{NT}})}
\]
• CONCISMIX Weight of the internal standard in the mix
• CONCNTMIX Weight of the neurotransmitter in the mix
• CONCNTSAMPLE Weight of the neurotransmitter in the sample
• PHNTMIX Peak height of the neurotransmitter in the mix
• PHISMX Peak height of the internal standard in the mix
• PHISSAMPLE Peak height of the internal standard in the sample

Concentration was in ng/20μl, as per volume injected. This was then converted to ng/ml of homogenised tissue. In order to determine the neurotransmitter concentration within each brain region, all neurotransmitter data was expressed as ng/g of tissue.

2.9 Statistical analysis

PAWS 18 statistical program and GraphPad Prism 5 was used to analyse all data. All data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene’s tests, respectively. Parametric data were assessed using Student’s unpaired t-test, two- or three-way analysis of variance (ANOVA), or repeated measures ANOVA to assess changes over time. Fisher’s LSD or Duncan’s post-hoc analysis was performed following ANOVA where appropriate. Non-parametric data were assessed using Kruskal-Wallis to determine overall differences between groups, Friedman’s test to assess changes over time, followed by Mann-Whitney U post-tests where appropriate. Pearson’s (for parametric data) and Spearman’s (for non-parametric data) correlation analyses were used to assess correlations between behavioural responding and levels of monoamines and/or neuroimmune mediators in the CNS. The level of significance was set at P ≤ 0.05. All data were expressed as the mean ± standard error of the mean (SEM).
Chapter 3

The effect of a depressive-like phenotype on nociceptive responding prior to and following peripheral nerve injury in the OB rat: determining alterations in monoamines and neuroinflammatory mediators
3.1 Introduction

As reviewed in Chapter 1, the clinical co-morbidity of depression and pain is widely recognised, with up to 80% of patients suffering from both disorders (Aguera-Ortiz et al., 2013, Bair et al., 2003, Gameroff and Olfson, 2006, Vaccarino et al., 2009, Poole et al., 2009). This co-morbidity has been associated with a heavier disease burden, increased disability and poor treatment response (Arnow et al., 2006). In contrast, sensitivity of depressed patients to experimental pain is controversial, with reports of decreased (Bar et al., 2007, Schwier et al., 2010), increased (Strigo et al., 2008a, Strigo et al., 2008b, Chiu et al., 2005) and unchanged (Giesecke et al., 2005) sensitivity to noxious and innocuous stimuli when compared to non-depressed controls. As such, greater understanding of the interaction between depression and pain is warranted.

In order to examine the neurobiological substrates mediating the depression-pain syndrome, it is necessary to combine animal models of depression with models of chronic pain. Depressive-like behaviour has been repeatedly observed in several animal models of chronic pain (Hu et al., 2009, Wang et al., 2011, Fukuhara et al., 2011, Suzuki et al., 2007). On the contrary, considerably less investigation has been conducted on nociceptive responding in animal models of depression. The current study aimed to examine nociceptive responding in an animal model of depression, prior to and following the induction of neuropathic pain. Clinical neuropathic pain represents approximately one in six cases of chronic pain (Torrance et al., 2006, Bouhassira et al., 2008, Smith and Torrance, 2012), arises from damage or lesion of the nervous system, and is a chronic, intractable condition that is often resistant to treatment (Dworkin et al., 2007). Neuropathic pain is associated with a lifetime prevalence of mood disorders of the order of 50% (Radat et al., 2013). Peripheral nerve injury in rodents mimics neuropathic pain in humans and models the somatic components of pain - allodynia (pain to a normally innocuous stimulus) and hyperalgesia (enhanced pain to a painful stimulus), and the affective components of pain.
However, conflicting data exist on whether neuropathic pain responding is altered in the presence of a preceding depressive state. Assessment of the effects of a depressive phenotype on neuropathic pain behaviour has revealed that mechanical allodynia is enhanced in Wistar-Kyoto rats (Zeng et al., 2008a) and following chronic restraint stress (Norman et al., 2010b) or social isolation (Norman et al., 2010a). In comparison, chronic constriction injury (CCI)-induced cold and mechanical allodynia is not altered following chronic mild stress or social isolation, however the affective pain behaviour was enhanced in these models (Bravo et al., 2013, Bravo et al., 2012). The olfactory bulbectomised (OB) rodent is a well-validated model of chronic depression (Willner and Mitchell, 2002), in our laboratory (Roche et al., 2007, Burke et al., 2010, Roche et al., 2012) and others (Cairncross et al., 1977, van Riezen and Leonard, 1990, Stock et al., 2000). Removal of the olfactory bulbs induces a constellation of behavioural, neurotransmitter, neuroendocrine and immune changes resembling those reported in depressed patients (Kelly et al., 1997, Song and Leonard, 2005). Reduced olfactory sensitivity has been observed in depressed patients (Pause et al., 2001, Pollatos et al., 2007), and antidepressant treatment enhances olfaction in patients (Gross-Isseroff et al., 1994). Furthermore, patients with acute depression demonstrate reduced olfactory bulb volume, an effect negatively correlated with depressive symptoms (Negoias et al., 2010). One of the most reliable behavioural outputs in bulbectomised rats is an increase in locomotor activity in a stressful, novel environment such as the open field. This hyperactivity is thought to reflect a lack of normal defensive behaviours and disinhibition of the amygdala (Leonard and Tuite, 1981, Harkin et al., 2003). Behavioural deficits in the OB rat are not thought to be related to anosmia, as peripheral ablation of olfaction does not result in the same behavioural changes (van Rijzingen et al., 1995, Calcagnetti et al., 1996). Removal of the bulbs causes retrograde and anterograde degeneration of cortico-limbic circuits, pathways which process not only emotion/affect, but also nociception. OB-induced behavioural changes include anhedonia, decreased social behaviour, learning and memory deficits, novelty-induced hyperactivity and reduced
sexual behaviour, which are selectively reversed by chronic, but not acute, antidepressant treatment (Kelly et al., 1997, Song and Leonard, 2005). Thus, this model is considered one of the most useful models currently available for the detection of antidepressant activity (Song and Leonard, 2005). The current literature indicates that OB rodents exhibit altered nociceptive responding. OB rats exhibit alterations in somatic nociceptive responding such as mechanical allodynia/hyperalgesia and thermal hyperalgesia (Rodríguez-Gaztelumendi et al., 2006, Burke et al., 2010). Furthermore, data from our laboratory indicates that OB rats exhibit enhanced nociceptive responding in the formalin test of tonic, persistent, inflammatory pain (Burke et al., 2010), which has been confirmed by others (Wang et al., 2010b). In addition, OB rats exhibit hyperalgesia following electrical stimulation of the dura mater surrounding the superior sagittal sinus, a model of trigeminovascular nociception (Liang et al., 2011). However, neuropathic pain responding has not been evaluated in the OB model. A number of procedures have been developed to investigate neuropathic pain processing in rodents, one of which is spinal nerve ligation (SNL) (Kim and Chung, 1992). SNL involves ligation of the L5 and L6 nerves distal to the dorsal root ganglia and results in allodynia and hyperalgesia to mechanical, cold and heat stimuli (Kim and Chung, 1992) and affective pain behaviour (LaBuda and Fuchs, 2005). Neuropathic pain-related behaviour develops within 24 to 48 hours of SNL surgery and has been shown to persist for between 10 to 16 weeks (Choi et al., 1994). As SNL does not appear to cause significant motor deficits (Hu et al., 2010, Kim and Chung, 1992, Chung et al., 2004) and the most commonly measured behavioural output in OB rats is locomotor activity, we proposed to examine nociceptive responding prior to and following SNL surgery in the OB model of depression.

A wealth of evidence has demonstrated a role for monoamines and inflammatory mediators in affective and nociceptive responding, although few studies have investigated the role of these mediators in the interaction between these processes. The dual role of monoamines in mood and pain is illustrated by the use of antidepressant drugs for the treatment of chronic neuropathic pain (Mico et al., 2006). As discussed in Chapter 1, the
monoaminergic projections that modulate mood and pain overlap significantly, and dysregulation of these pathways may lead to depression and enhanced pain perception. The synthesis, release, and reuptake of the monoamines can be modulated by inflammatory cytokines (Miller, 2009). Indoleamine 2,3-dioxygenase 1 (IDO1) is a rate-limiting enzyme in tryptophan metabolism which is upregulated in response to inflammation, resulting in decreased serotonin content and depressive-like behaviour (Popov et al., 2006, Heyes et al., 1992, O'Connor et al., 2009a, Dantzer et al., 2008). Recently, it was reported that depressive-like behaviour in rats with chronic inflammatory pain was associated with increased IDO1 and IL-6 mRNA with a concurrent reduction of serotonin/tryptophan in the hippocampus, and that IDO and IL-6 are increased in the plasma of patients with chronic pain and depression (Kim et al., 2012). Therefore, monoamines such as serotonin that have well-known roles in depression and pain may be depleted via increased inflammation. Neuroinflammatory processes are now well-recognised to play important roles in the pathophysiology of depression and chronic pain. For example, administration of interleukin (IL)-6 into the amygdala or hippocampus increases immobility in the forced swim test (Wu and Lin, 2008). Chronic stress-induced anhedonia is mediated by hippocampal IL-1β (Koo and Duman, 2008) and central IL-1β plays a key role in chronic stress-induced depressive phenotypes (Goshen et al., 2008). Neuroinflammation is critically involved in neuropathic pain. Previous studies have shown elevations in microglial and astrocyte activation in the spinal cord following SNL (Wang et al., 2009b, Jin et al., 2003), however it is not known whether spinal cord glial activation following SNL is altered in a model of depression. In addition, although it is established that spinal inflammatory processes are essential for the development of neuropathic pain (Vallejo et al., 2010), the role of neuroimmune mediators in the supraspinal processing of pain is less understood. Reserpine-induced depression-pain syndrome is associated with enhanced nitrosative stress and inflammatory cytokines in the cortex and hippocampus (Arora et al., 2011) and central administration of IL-1ra ameliorates the effects of neuropathic pain on depressive behaviour.
Thus, monoaminergic changes, glial activation and pro-inflammatory cytokines in discrete brain regions are involved in both affective and nociceptive processing, and may be responsible for the altered nociceptive responding associated with depression.

Disruptions of the monoaminergic and immune systems have been demonstrated in OB rats. Serotonergic deficits and decreased turnover in the frontal cortex, nucleus accumbens, hypothalamus, hippocampus, amygdala and mid-brain (Lumia et al., 1992, Song and Leonard, 1995, Redmond et al., 1997, Connor et al., 1999, Hellweg et al., 2007), and reduced noradrenaline and dopamine in the frontal cortex (Redmond et al., 1997, Redmond et al., 1999, Xu et al., 2005) have been reported. Furthermore, previous work from our laboratory demonstrated that enhanced inflammatory hyperalgesia in OB rats was associated with reduced 5-hydroxyindoleacetic acid (5-HIAA) concentration in the hippocampus and amygdala (Burke et al. 2010).

Moreover, OB rats have been shown to exhibit increased circulating IL-1β and prostaglandins (Song et al., 2009), a blunted inflammatory response to LPS (Connor et al., 2000), and increased IL-1β, TNFα, prostaglandins and GFAP in the brain (Rinwa et al., 2013, Borre et al., 2012b, Song et al., 2009, Cizkova et al., 1997) making it an appropriate model to investigate monoaminergic and immune alterations that may be implicated in depression and pain. As such, a further aim of the current study was to determine if interactions between OB and SNL at a behavioural level are associated with concomitant interactions at the monoaminergic and neuroimmune level.
Chapter 3 – Characterising neuropathic pain in the OB rat

3.1.1 Hypothesis and Aims

**Hypothesis**
Nociceptive, neuropathic and affective pain behaviour would be enhanced in the OB model of depression, an effect that would be accompanied by alterations in neuroimmune mediators and/or monoamine levels in the CNS.

1. Characterise nociceptive responding to mechanical, heat and cold stimuli prior to and following peripheral nerve injury in the OB rat model of depression
2. Investigate if affective pain-related behaviour following SNL is altered in the OB rat
3. Investigate if OB-related hyperactivity on exposure to the open field persists following SNL
4. Examine if SNL-induced glial activation at the level of the spinal cord is altered in the OB model of depression.
5. Examine if serum corticosterone levels are altered by OB, SNL and their interaction.
6. Determine if interactions between OB and SNL at a behavioural level are accompanied by alterations at the neuroimmune and monoaminergic level in discrete brain regions responsible for the processing of emotion and pain (PFC, amygdala, thalamus and hippocampus).
3.2 Materials & Methods

3.2.1 Animal husbandry

Male Sprague Dawley rats (Charles River, Margate, UK) weighing 175-200g on arrival were housed singly in plastic-bottomed cages (45 × 25 × 20 cm) containing wood shavings as bedding, in a temperature controlled room (21 ± 2°C), with a 12:12h light-dark cycle (lights on at 0700h). Rats were fed a standard laboratory diet; food and water were available ad libitum. Baseline testing began 5 days following arrival of rats to the unit and all testing was carried out during the light phase. The experimental protocol was carried out in accordance with the guidelines and approval of the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609 and every effort was made to minimise the number of animals used and their suffering.

3.2.2 Experimental procedure

The experimental design is presented in Figure 3.1. Essentially, male Sprague Dawley rats were tested in the open field (Section 2.4.1.1), von Frey test (Section 2.4.2.1), Hargreaves test (Section 2.4.2.2) and the acetone drop test (Section 2.4.2.3) in order to determine baseline locomotor and nociceptive responding, following which animals were randomly assigned to either sham (n=18) or olfactory bulbectomy (OB) (n=20) surgery groups (Section 2.2). Two weeks following surgery, animals were re-tested in the aforementioned tests. Animals were subsequently randomly allocated to one of four groups: sham-non spinal nerve ligation (sham-NSNL) (n=8), OB-NSNL (n=7), sham-SNL (n=10) and OB-SNL (n=11) (Section 2.3). Mechanical withdrawal thresholds were examined on days 1, 5, 8, 12 and 15 post-SNL or NSNL surgery. Locomotor activity was examined on day 14. Animals were tested in the acetone drop test and Hargreaves test on day 19 and 20, respectively. Affective pain behaviour was examined in the place
escape/avoidance paradigm on day 21 (Section 2.4.2.4). Twenty-four hours following the last behavioural assessment, animals were sacrificed by decapitation (between 09:00 and 13:00h), trunk blood collected and the brain and spinal cord removed. Serum was collected and stored at -80°C until enzyme immunoassay for corticosterone levels was carried out (Section 3.2.2). Discrete brain regions were dissected out on an ice cold plate and stored at -80°C until quantitative RT-PCR analysis (Section 2.7) was performed for the expression of inflammatory mediators and HPLC with electrochemical detection (Section 2.8) for levels of monoamines. The spinal cord was washed in ice-cold sterile PBS, placed in 4% paraformaldehyde for twenty-four hours followed by immersion in 20% sucrose-azide until processed immunohistochemically for markers of astrocyte and microglial activation (Section 3.2.3).

3.2.3 Corticosterone enzyme immunoassay (EIA)

Quantification of corticosterone in serum samples was performed with the Corticosterone EIA Kit according to the manufacturer’s instructions (Item no. 500655, Cayman Chemical Company, Ann Arbor, MI, USA). Serum samples were defrosted on ice and spun at 14,000g at 4°C for 15 minutes. In a 96-well plate, 100µl of EIA buffer was added to Non-Specific Binding (NSB) wells, and 50µl of EIA buffer was added to Maximum Binding (B0) wells. 50µl of S1-S8 corticosterone EIA standards (5,000, 2,000, 800, 320, 200, 80, 40, 20, 10, 5, 2, 1) were added to the wells, and the plate was incubated for 1 hour at room temperature. Then, 50µl of tracer was added to each well, and the plate was incubated for 1 hour at room temperature. After washing, 50µl of substrate solution was added to each well, and the plate was incubated for 15 minutes at room temperature. Finally, 50µl of stop solution was added to each well, and the absorbance was measured at 450nm. The concentration of corticosterone in the serum samples was calculated using the standard curve.

Fig. 3.1 Experimental Protocol Abbreviations: NSNL non-Spinal Nerve Ligation; OB Olfactory bulbectomy; PEAP Place escape/avoidance paradigm; SNL Spinal Nerve Ligation
Chapter 3 – Characterising neuropathic pain in the OB rat

128, 51.2, 20.5, and 8.2 pg/ml) were added in duplicate into corresponding wells on the plate, and 50µl of each sample was added in duplicate into corresponding wells. 50µl of Corticosterone AChE Tracer was added to each well except the Total Activity (TA) and the Blank (Blk) wells, and 50µl of Corticosterone EIA Antiserum was added to each well except the TA, Blk, and NSB wells. The plate was covered with plastic film and incubated for two hours at room temperature on an orbital shaker, after which wells were washed five times with wash buffer. Thereafter, 200µl of Ellman’s Reagent was added to each well, and 5µl of tracer to the TA well. The plate was covered with plastic film and incubated on an orbital shaker in the dark to allow optimum development. The plate was read at a wavelength of 412nm after 60 minutes. Calculations and data analysis were performed by subtracting the blank reading and NSB from all samples to get the corrected B0, and the concentration of corticosterone in samples was determined from the standard curve using a non-linear regression calculated in GraphPad Prism. The detection limit for the assay was 30pg/ml.

3.2.4 Immunohistochemical determination of microglia and astrocytes in the spinal cord

Microglia (OX42) and astrocyte activation (GFAP) was determined in the L5 dorsal horn of the spinal cord using immunohistochemistry. After equilibration in 20% sucrose-azide solution, 30µm transverse spinal cord sections were cut using a cryostat and immunohistochemical staining was performed as previously described (Walsh et al., Roche et al., 2007). Free-floating sections were placed in Greiner pots, washed 3 times for 5 minutes in tris-buffered saline (TBS), and then quenched in 0.75% hydrogen peroxide (H2O2) for 15 minutes to remove endogenous peroxidase activity, washed again and subsequently placed in TBS, 0.2% Triton-X (Tx) containing 3% normal horse/goat serum for 1hr to prevent non-specific binding. Sections were then incubated in the primary antibody [mouse anti-OX42 (1:400; Chemicon, Watford, UK [CBL1512]) or rabbit anti-GFAP (1:2000; DAKO, Stockport, UK [Z0334]), diluted in TBS-Tx with 1% normal horse/goat serum overnight at room temperature. The sections were
then incubated with the biotinylated antisera (1:200) (horse anti-mouse [BA-2001]; Vector, Peterborough, UK) or goat anti-rabbit (11-065-144; Jackson Immunoresearch, Suffolk, UK), followed by streptavidin-biotin-horseradish peroxidase (Vector, Peterborough, UK). Immunolabelling was revealed by incubating the sections in a 0.05% solution of diaminobenzidine tetrahydrochloride in TBS containing 0.003% hydrogen peroxide. Sections were mounted on gelatin-coated microscope slides, dehydrated in an ascending series of alcohols, cleared in xylene and coverslipped using DPX mounting medium.

3.2.4.1 Quantification of immunohistochemical staining

Photomicrographs of the L5 dorsal horn were taken with an Olympus microscope BX40 and Olympus C5060 digital camera (Mason Technology, Dublin, Ireland) by a researcher blinded to group identity. Immunoreactivity (IR) for OX42 (marker of microglial activation) and GFAP (marker of astrocyte activation) was quantified by comparing the average intensity of staining from ipsilateral and contralateral grey matter obtained from digitized images of 3 to 5 random intact L5 sections per animal as determined by the rat brain atlas. The digitized images were captured from a fixed area under 4× magnification bilaterally and quantified as mean grey value (MGV) for the region of interest (ROI) and background (BR) using Image J software (National Institute of Mental Health, NIMH). Specifically, the MGV from an area containing the grey matter of the upper dorsal horn was obtained by outlining the most dorsal aspect of the dorsal horn to the start of the neck, as previously described (Coyle, 1998) (Fig 3.2). Optical density (OD) was calculated as $\text{LOG}_{10}(255/\text{MGV}_{\text{ROI}}) - \text{LOG}_{10}(255/\text{MGV}_{\text{BR}})$.

Fig. 3.2 Schematic representing the area of the dorsal horn of the L5 spinal cord where quantification of microglia and astrocytes was performed.
3.2.5 Statistical analysis

PAWS 18 statistical program was used to analyse all data. Parametric data were assessed using Student’s unpaired t-test, two-way analysis of variance (ANOVA) using factors of bulbectomy and SNL, or repeated measures ANOVA to assess changes over time. Fisher’s LSD *post-hoc* analysis was performed following ANOVA where appropriate. Non-parametric data was assessed using Kruskal-Wallis to determine overall differences between groups, followed by Mann-Whitney U post-tests where appropriate. Pearson’s (for parametric data) and Spearman’s (for non-parametric data) correlation analyses were used to assess correlations between nociceptive thresholds and the expression of inflammatory mediators in the brain and spinal cord. The level of significance was set at $P \leq 0.05$. All data were expressed as the mean ± standard error of the mean (SEM).
3.3 Results

3.3.1 Behaviour in the open field or nociceptive responding did not differ between groups assigned to sham or OB prior to surgery (Table 3.1).

<table>
<thead>
<tr>
<th>Behavioural test</th>
<th>Sham</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open field</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance moved (cm)</td>
<td>2530±75</td>
<td>2706±57</td>
</tr>
<tr>
<td>Time in inner zone (s)</td>
<td>18±5</td>
<td>18±3</td>
</tr>
<tr>
<td><strong>Acetone drop test</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Latency to respond (s)</td>
<td>47±3</td>
<td>45±4</td>
</tr>
<tr>
<td>Withdrawal frequency</td>
<td>1±0.6</td>
<td>2±0.4</td>
</tr>
<tr>
<td><strong>von Frey test</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical threshold (EC$_{50}$) (g)</td>
<td>10±1</td>
<td>9±0.6</td>
</tr>
<tr>
<td>Lowest filament at which response elicited (g)</td>
<td>5.5±0.5</td>
<td>5±0.4</td>
</tr>
<tr>
<td><strong>Hargreaves test</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Latency to respond (s)</td>
<td>9.7±0.8</td>
<td>9.5±0.7</td>
</tr>
</tbody>
</table>

Table 3.1. Baseline behaviour prior to sham/OB surgery. Data presented as mean ± SEM, n=18-20

3.3.2 Effect of OB and SNL on body weight

Two-way repeated measures ANOVA revealed an effect of OB ($F_{(1,25)} = 8.23$, $P=0.008$), time ($F_{(5,125)} = 478.92$, $P<0.001$) and a time × OB interaction ($F_{(5,125)} = 4.84$, $P<0.001$) on body weight measured at the time points shown (Fig. 3.3). Body weights did not differ prior to surgery and all animals gained weight over the study period (Fig. 3.3). SNL surgery did not alter body weight in either sham or OB animals. However, OB animals exhibited lower body weight compared to sham controls up to day 14 post-OB surgery, with a similar tendency observed following SNL (Fig. 3.3)

![Fig. 3.3 Body weight over the study period **P<0.01 vs. Sham](image)
3.3.3 OB rats exhibit increased locomotor activity in the open field

To confirm depressive-like behaviour in the OB rat, locomotor activity was examined 14 days following sham/OB surgery in the open field. Repeated measures two-way ANOVA revealed an effect of OB ($F_{(1,34)} = 25.17$, $P<0.001$), time ($F_{(4,136)} = 8.89$, $P<0.001$), and a time × OB interaction ($F_{(4,136)} = 5.7$, $P<0.001$) on distance moved per minute over the 5 minute trial period in the open field. Post-hoc analysis revealed that OB animals demonstrated a characteristic hyperactivity response on exposure to the open field test, expressed as an increase in distance moved when compared to sham-operated counterparts ($t_{34} = 5.02$, $P<0.001$, Fig. 3.4 A&B). Anxiety-like behaviour was assessed in the model by examining time spent in the inner zone of the open field. OB animals demonstrated a tendency to spend less time in the inner zone, although this failed to reach statistical significance ($t_{32} = 1.81$, $P=0.08$, Fig. 3.4C). There was a tendency of OB rats to exhibit increased defecation, suggestive of increased emotionality/anxiety-like behaviour ($U = -1.71$, $P=0.084$; Fig 3.4D). General behaviours such as time spent rearing or grooming did not significantly differ between sham and OB rats (Fig.3.4 E&F)
Fig. 3.4 Locomotor activity of sham and OB rats in the open field 14 days after surgery (A) Distance moved over the 5 minute period (B) Total distance moved (C) Time in the inner zone of the open field (D) Faecal pellet count in the open field (E) Rearing duration (F) Grooming duration. Data presented as mean ± SEM, n=18-20
3.3.4 OB rats exhibit increased nociceptive responding to mechanical and cold, but not heat, stimuli

The effect of bulbectomy on nociceptive responding to mechanical and thermal stimuli was examined following establishment of the depressive-like behavioural phenotype in these animals. OB rats developed mechanical allodynia ($t_{34} = 2.71, P=0.011$) as evidenced by a significant reduction in the 50% mechanical withdrawal threshold and responded at lower von Frey filament forces when compared to sham-operated controls ($U = 61.5, P=0.001$ Fig. 3.5 A&B). OB rats exhibited cold allodynia, expressed as a decrease in the latency to respond (lick, shake or withdraw the hind-paws) ($t_{34} = 2.15 P = 0.039$) and a trend towards an increase in the withdrawal frequency following the application of acetone to the plantar surface of the hind-paws ($U = 103.5, P=0.06$), when compared to sham-operated controls (Fig. 3.5 C&D). In the Hargreaves test, there was no significant effect of OB on the latency to respond to a noxious heat stimulus applied to the hind-paws (Fig. 3.5E).
Fig. 3.5 Basal nociceptive responding of sham and OB rats 16-18 days post-surgery
(A) 50% mechanical withdrawal thresholds and (B) the lowest filament force to elicit a
response in the von Frey test. (C) Paw withdrawal latency and (D) withdrawal frequency
following the application of acetone to the hind-paws. (E) Latency to withdraw from a
noxious heat stimulus applied to the hind-paws in the Hargreaves test. *P<0.05,
**P<0.01 vs. sham-operated controls. Data expressed as mean ± SEM, n=18-20
3.3.5 OB rats demonstrate altered nociceptive responding to mechanical and cold stimuli following SNL surgery

The effect of SNL on nociceptive responding to mechanical, cold and heat stimuli in sham and OB animals was examined. Repeated measures two-way ANOVA revealed a significant effect of OB (F(1,33) = 15.92, P<0.001), time (F(5,165) = 12.13, P<0.001), side of injury (F(1,33) = 16.22, P<0.001) and time × side interaction (F(5,165) = 6.05, P<0.001) on 50% mechanical withdrawal thresholds following SNL surgery. *Post-hoc* analysis revealed that SNL resulted in significantly decreased mechanical thresholds of the ipsilateral (left) hind-paw in both sham and OB animals at all post-injury time points when compared to pre-SNL levels, indicating SNL-induced mechanical allodynia (Fig. 3.6A). There was no difference in mechanical thresholds of the ipsilateral hind-paw between sham or OB animals at any of the time points examined following SNL (sham-ipsi vs. OB-ipsi). Mechanical thresholds of the contralateral (right) hind-paw were not altered by SNL surgery in sham animals (Fig. 3.6A). In comparison, OB animals exhibited reduced mechanical thresholds of the contralateral hind-paw from day 5 through day 15 post-SNL surgery when compared to sham-operated controls (sham-contra vs. OB-contra; Fig. 3.6A). Analysis of the area under the curve revealed an effect of OB (F(1,38) = 11.89, P=0.001) and side of injury (F(1,38) = 24.14, P<0.001) on mechanical thresholds. *Post-hoc* analysis revealed that SNL reduced mechanical thresholds of the ipsilateral hind-paw of both sham and OB animals when compared to their respective contralateral side (Fig. 3.6B). In addition, OB-SNL rats exhibited reduced mechanical thresholds of the contralateral hind-paw when compared to sham-SNL counterparts. Thus, the above data demonstrate that while SNL results in mechanical alldynia of the ipsilateral hind-paw of sham animals, OB animals exhibit bilateral mechanical alldynia following peripheral nerve injury.

Kruskal-Wallis test revealed an overall effect of group for the latency of the hind-paw to withdraw following acetone application ($\chi^2_{(3)} = 15.23$, P=0.002). SNL resulted in decreased paw withdrawal latency of the ipsilateral hind-paw of both sham and OB animals, when compared to their
contralateral side, indicating SNL-induced cold allodynia (Fig. 3.6C). In addition, OB-SNL animals exhibited noticeably lower paw withdrawal latencies of the ipsilateral hind-paw when compared to sham-SNL counterparts, an effect which just failed to reach significance (U = -1.91, P=0.056).

Kruskal-Wallis test revealed an overall effect of group for the frequency of hind-paw withdrawals following acetone application ($\chi^2(3) = 9.032$, P=0.029). SNL resulted in increased withdrawal frequency of the ipsilateral hind-paw of both sham and OB animals, when compared to their contralateral side, indicative of SNL-induced cold alldynia (Fig. 3.6D). OB did not alter the withdrawal frequency of either the ipsi- or contra-lateral hind-paw when compared to sham animals. There was no effect of OB or side of injury on latency to respond to a noxious heat stimulus in the Hargreaves test (Fig. 3.6E).

There was no difference in withdrawal thresholds of the ipsilateral vs. contralateral hind-paw of NSNL animals (see appendix Fig. B.1). OB-NSNL animals exhibited lower thresholds of the contralateral hind-paw when compared to sham-NSNL controls, with a similar trend observed in the ipsilateral hind-paw, demonstrating OB-induced mechanical allodynia.
Fig. 3.6 Nociceptive responding of sham and OB rats to mechanical and thermal (heat and cold) stimuli following SNL surgery (A) Mechanical withdrawal thresholds of the ipsi- and contra-lateral hind-paw of sham and OB animals prior to and following SNL surgery (B) Area under the curve of mechanical withdrawal thresholds (C) Paw withdrawal latency and (E) withdrawal frequency to acetone application to the hind-paw following SNL (E) Paw withdrawal latency in the Hargreaves test following SNL. ++P<0.01 Sham-ipsi and OB-ipsi vs. pre-SNL levels, *P<0.05 OB ipsi/contra vs. Sham-ipsi/contra, δP<0.05 OB-contralateral vs. Sham-contralateral side. Data expressed as mean ± SEM, n=7-11.
Due to the differences in nociceptive responding of sham and OB rats prior to SNL, behavioural data obtained following SNL was also examined by expressing data as percentage of pre-SNL levels. Analysis of normalised thresholds revealed that SNL resulted in mechanical and cold allodynia, but not heat hyperalgesia, of the ipsi- but not contra-lateral hind-paw of both sham and OB rats (Figure 3.7 A-B when compared to the dotted line representing pre-SNL levels). OB rats exhibited altered nociceptive responding to acetone application (cold), but not mechanical or heat stimuli following SNL, exemplified by the reduced paw withdrawal latency ($t_{20} = 2.76, P=0.019$) and decreased number of responses ($t_{19} = 3.21, P=0.005$) of the ipsilateral hind-paw, when compared to sham-SNL controls (OB-SNL vs. sham-SNL, Fig. 3.7A). There was no significant difference between sham and OB rats in the withdrawal threshold of the contralateral hind-paw to mechanical, cold or heat stimuli following SNL (Fig. 3.7B).

**Fig 3.7 Nociceptive responding of sham and OB rats to mechanical and thermal stimuli following SNL surgery normalised to pre-SNL levels.** Mechanical, cold (acetone) and heat thresholds of the (A) ipsi- and (B) contra-lateral hind-paw of sham and OB rats following SNL expressed as a percentage of pre-SNL levels. Mechanical responding calculated as an average over post-SNL testing period. *$P<0.05$, **$P<0.01$ vs. sham-operated controls, dotted line represents pre-SNL levels. Data presented as mean ± SEM, N=7-11
### 3.3.6 SNL induces anxiety-like behaviour in the open field in sham, but not OB, animals

Two-way ANOVA revealed an overall effect of OB on total distance moved in the open field 14 days following NSNL/SNL surgery ($F_{(1,32)} = 4.41$, $P=0.044$, Fig. 3.8A). SNL did not alter distance moved in the open field, but resulted in a decrease in time spent in the inner zone ($F_{(1,31)} = 4.83$, $P=0.036$) in sham animals, suggestive of SNL-induced anxiety-like behaviour (Fig. 3.8B). In comparison, OB rats exhibited a non-significant decrease in the time spent in the inner zone, an effect which may have masked any further reduction by SNL. Kruskal-Wallis revealed an overall effect of group for faecal pellet count ($\chi^2_{(3)} = 11.41$, $P=0.01$). OB animals demonstrated significantly increased defecation in the open field compared to shams (OB-NSNL vs. sham-NSNL, Fig. 3.8C), an effect which just failed to reach significance following SNL ($P=0.08$). Grooming, but not rearing, behaviour was significantly increased in OB-NSNL animals when compared to sham counterparts (OB: $F_{(1,22)} = 6.83$, $P=0.016$, Fig. 3.8E), an effect not seen following SNL.
Fig. 3.8 Locomotor activity of sham and OB animals 14 days following NSNL/SNL surgery (A) Total distance moved in the open field 14 days after NSNL/SNL surgery (B) Time in the inner zone of the open field 14 days after NSNL/SNL surgery. (C) Pellet count (D) Rearing duration (E) Grooming duration. αP<0.05 effect of OB (ANOVA), *P<0.05, **P<0.01 vs. Sham-NSNL. Data expressed as mean ± SEM, n=7-11.
3.3.7 **OB animals demonstrate reduced time in the light chamber of the place escape/avoidance paradigm**

Repeated measures two-way ANOVA revealed a significant effect of OB ($F_{(1,29)} = 20.72$, $P<0.001$), SNL ($F_{(1,29)} = 9.77$, $P=0.004$), time ($F_{(5,145)} = 11.07$, $P<0.001$), and a time $\times$ OB interaction ($F_{(5,145)} = 2.89$, $P=0.016$) on time spent in the light-side of the place escape/avoidance paradigm (PEAP) chamber over the 30 minutes of the test (Fig 3.9A). Post-hoc analysis revealed that sham-SNL animals exhibited reduced time in the light chamber from the 5\textsuperscript{th} - 30\textsuperscript{th} minute when compared to NSNL counterparts. OB-NSNL animals spent less time in the light-side of the PEAP chamber for most of the duration of the trial (time bin 1-5; 0-25 min) when compared to sham-NSNL controls. Two-way ANOVA of the total time in the light-side of the arena over the 30 minute period revealed a significant effect of OB ($F_{(1,29)} = 20.88$, $P<0.0001$) and SNL ($F_{(1,29)} = 9.82$, $P=0.004$, Fig 3.9B). OB resulted in decreased time in the light-side of the chamber over the entire 30 minute trial when compared to sham counterparts. SNL animals exhibited a decrease in time in the light-side of the chamber (sham-NSNL vs. Sham-SNL). OB-SNL animals exhibited a further reduction in the amount of time spent in the light-side of the chamber when compared to sham-SNL counterparts, indicating a potential additive effect of OB and SNL in this test (Fig. 3.9B).
Fig. 3.9 Behaviour in the place escape/avoidance paradigm 21 days after NSNL/SNL surgery (A) Time in the light chamber over the 30 minute period. (B) Total time in the light chamber. *P<0.05, **P<0.01 vs. sham-NSNL, †P<0.05 vs. sham-SNL. Data expressed as mean ± SEM, n=7-11.
3.3.8 SNL increased serum corticosterone levels in both sham and OB animals

SNL resulted in increased serum corticosterone on day 22 after surgery (two-way ANOVA effect of SNL $F_{(1,26)} = 4.76$, $P=0.038$, Fig. 3.10), an effect not altered by OB.

Fig. 3.10 Serum corticosterone (CORT) levels in sham and OB rats following NSNL/SNL surgery. $^\& P<0.05$ effect of SNL (ANOVA). Data expressed as mean ± SEM, $n=7-11$. 
3.3.9 OB rats exhibit increased astrocyte activation in the dorsal horn of the spinal cord following SNL: inverse correlation between mechanical thresholds and astrocyte immunostaining

GFAP and OX42 immunoreactivity in the dorsal horn of the L5 spinal cord was examined to determine if OB altered expression of astrocytes and microglia, respectively, following NSNL or SNL surgery. Immunohistochemical analysis revealed that SNL induced an increase in OX42 immunoreactivity (Two-way ANOVA effect of SNL: $F_{(2,20)} = 16.04 \ P = 0.001$) in the ipsilateral, but not contralateral dorsal horn of the spinal cord of both sham and OB rats (sham/OB-SNL vs. sham/OB-NSNL, Fig 3.12 B-E), suggesting that SNL induces increased microglial activation in the spinal cord of both sham and OB animals. GFAP immunoreactivity was increased in the ipsilateral and contralateral (OB × SNL interaction: $F_{(1,21)} = 5.93 \ P = 0.024$) dorsal horn of OB-SNL rats when compared to NSNL controls (OB-SNL vs. OB-NSNL, Fig 3.12 F-I), indicating bilateral astrocyte activation in OB-SNL animals. Pearson’s correlation analysis revealed an inverse correlation between GFAP immunoreactivity in the dorsal horn of the spinal cord and AUC for mechanical thresholds post SNL ($r^2 = 0.2337 \ P=0.019$, Fig. 3.11). No other significant correlations between immunoreactivity and behaviour were observed.

![Figure 3.11](image)

**Fig. 3.11** Pearson’s correlation analysis revealed a significant inverse correlation between GFAP immunoreactivity in the dorsal horn of the spinal cord and AUC for mechanical thresholds post SNL.
Chapter 3 – Characterising neuropathic pain in the OB rat

Fig 3.12 Microglial and astrocyte immunostaining in the L5 spinal cord following NSNL/SNL. (A) Schematic depicting area of dorsal horn analysed for immunohistochemical labelling. (B-C) Representative photomicrographs of OX42 immunostaining in the ipsi- and contra-lateral L5 dorsal horn of sham-SNL rats. (Bi) high magnification of OX42 staining of boxed area in (B). (D-E) OX42 immunostaining in the dorsal horn of sham and OB rats following NSNL/SNL. (F-G) Representative photomicrographs of GFAP immunostaining in the ipsi- and contra-lateral L5 dorsal horn of sham-SNL rats. (Fi) high magnification of GFAP staining of boxed area in (F). (H-I) GFAP immunostaining in the dorsal horn of sham and OB rats following NSNL/SNL. Data presented as mean ± SEM. *P<0.05 **P<0.01 vs. NSNL. Scale bar = 100µm (B,C,F,G) or 20µm (Bi and Fi).
3.3.10 OB- and SNL-induced changes in the expression of glial markers and cytokines in discrete brain regions

The following brain regions investigated were chosen based on their role in the processing of emotion and pain. Analysis of cytokine expression in brain regions on both the right and left side revealed no significant lateralisation effects, thus data were pooled for subsequent analysis. Data were expressed as a percentage of sham-NSNL controls.

3.3.10.1 Effect of OB and/or SNL on the expression of neuroinflammatory mediators in the thalamus

Two-way ANOVA revealed effects of OB on CD11b (microglial marker) mRNA expression in the thalamus ($F_{(1,25)} = 4.54$, $P=0.043$). Post-hoc comparisons revealed that OB resulted in increased CD11b expression when compared to sham controls (OB-NSNL vs. sham-NSNL Fig 3.13 E-G), with a similar trend observed for GFAP ($F_{(1,27)} = 3.6$, $P=0.067$). OB did not alter the expression of cytokines when compared to sham animals (Sham-NSNL vs. OB-NSNL, Fig 3.13 D-G). Two-way ANOVA revealed an effect of SNL on CD11b ($F_{(1,25)} = 8.69$, $P=0.007$), CD40 ($F_{(1,26)} = 13.50$, $P=0.001$), GFAP ($F_{(1,27)} = 7.85$, $P=0.009$), TNFα ($F_{(1,27)} = 6.11$ $P = 0.02$), IL-6 ($F_{(1,26)} = 7.57$ $P = 0.01$) and IL-10 ($F_{(1,21)} = 4.93$ $P = 0.038$) expression in the thalamus. Post-hoc comparisons revealed that OB-SNL animals exhibited a decrease in CD11b, CD40, GFAP and TNFα mRNA expression and an increase in IL-6 mRNA expression when compared to NSNL counterparts (OB-NSNL vs. OB-SNL Fig 3.13).
Fig. 3.13 Glial markers and cytokine expression in the thalamus following OB and SNL. (A) CD40 (B) CD11b (C) GFAP (D) IL-1β (E) TNFα (F) IL-6 (G) IL-10. Data expressed as mean ± SEM. *P<0.05 vs. sham-NSNL, #P<0.05 vs. OB-NSNL, &P<0.05 effect of SNL (ANOVA). N=7-10
3.3.10.2 Effect of OB and/or SNL on the expression of neuroinflammatory mediators in the amygdala

In the amygdala, two-way ANOVA revealed an effect of OB on CD11b ($F_{(1,27)} = 6.11$, $P=0.02$) and IL-1β ($F_{(1,27)} = 27.4$, $P<0.001$) mRNA expression; an effect of SNL on IL-6 ($F_{(1,23)} = 21.4$, $P < 0.01$) and IL-10 ($F_{(1,24)} = 10.56$, $P = 0.003$) expression; and an OB × SNL interaction for CD11b ($F_{(1,27)} = 4.22$, $P=0.05$), GFAP ($F_{(1,27)} = 5.77$, $P=0.023$) and IL-1β ($F_{(1,24)} = 4.50$, $P = 0.04$) mRNA expression. Post-hoc comparisons revealed that OB-NSNL animals exhibited increased CD11b, GFAP and IL-1β mRNA expression when compared to sham-NSNL counterparts (Fig 3.14 B-D). The OB-associated increases in CD11b and IL-1β were significantly attenuated in the presence of SNL (OB-NSNL vs. OB-SNL, Fig 3.14 B&D). In addition, the OB-associated increase in GFAP expression was not observed following SNL surgery (OB-NSNL vs. OB-SNL, Fig 3.14C). There was no effect of OB or SNL on TNFα mRNA expression in the amygdala, however, SNL resulted in reduced mRNA expression of IL-6 in both sham and OB animals (Fig 3.14F) and increased expression of IL-10 (Fig. 3.14G) in sham, but not OB, animals.
Amygdala

Fig. 3.14 Glial markers and cytokine expression in the amygdala following OB and SNL. (A) CD40 (B) CD11b (C) GFAP (D) IL-1β (E) TNFα (F) IL-6 (G) IL-10. Data expressed as mean ± SEM. *P < 0.05, **P < 0.01 vs. sham-NSNL. ***P < 0.01 vs. OB-NSNL. N=7-10
3.3.10.3 Effect of OB and/or SNL on the expression of neuroinflammatory mediators in the hippocampus

There was no significant effect of OB on the expression of glial activation markers or cytokines in the hippocampus. Although ANOVA revealed a significant effect of SNL on IL-6 mRNA expression ($F_{(1,28)} = 10.20 \ P=0.01$, Fig 3.15F), post-hoc analysis failed to identify any significant differences in IL-6 expression between animals.

![Graphs showing the expression of neuroinflammatory mediators in the hippocampus](image)

**Fig. 3.15** Glial markers and cytokine expression in the hippocampus following OB and SNL. (A) CD40 (B) CD11b (C) GFAP (D) IL-1β (E) TNFα (F) IL-6 (G) IL-10. Data expressed as mean ± SEM. *P<0.05 effect of SNL (ANOVA) N=7-10
3.3.10.4 Effect of OB and/or SNL on the expression of neuroinflammatory mediators in the prefrontal cortex

Two-way ANOVA revealed an effect of OB on CD40 ($F_{(1,18)} = 5.12$, $P=0.036$) and GFAP ($F_{(1,18)} = 6.66$, $P=0.019$) mRNA expression; an effect of SNL on TNFα ($F_{(1,17)} = 19.50$ $P<0.01$) mRNA expression; and an OB × SNL interaction on IL-1β ($F_{(1,18)} = 4.85$ $P=0.04$) and IL-10 ($F_{(1,19)} = 4.96$ $P=0.038$) mRNA expression in the prefrontal cortex (PFC).

OB did not significantly alter the expression of inflammatory mediators in NSNL animals (OB-NSNL vs. sham-NSNL). In comparison, OB-SNL rats exhibited increased CD40, GFAP, IL-1β, and IL-10 mRNA expression when compared to sham-SNL counterparts (Sham-SNL vs. OB-SNL, Fig 3.16A-G). Furthermore, SNL resulted in increased mRNA expression of TNFα in OB, but not sham, rats when compared to NSNL counterparts (OB-NSNL vs. OB-SNL, Fig. 3.16F).
Fig. 3.16 Glial markers and cytokine expression in the PFC following OB and SNL. (A) CD40 (B) CD11b (C) GFAP (D) IL-1β (E) TNFα (F) IL-6 (G) IL-10. **P<0.01 vs. OB-NSNL. #P<0.05 vs. OB-NSNL. †P<0.05 vs. sham-NSNL. Data expressed as mean ± SEM. N=7-10
Spearman’s correlation analysis revealed a significant positive correlation between paw withdrawal latency in the acetone drop test following SNL surgery and IL-6 mRNA expression in the amygdala \((r = 0.620, P=0.0006, \text{Fig. 3.17A})\). Normalised withdrawal frequency of the ipsilateral hind-paw in the acetone drop test following SNL surgery was positively correlated with IL-10 expression in the amygdala \((r = 0.381, P=0.046 \text{ Fig. 3.17B})\) and negatively correlated with IL-10 expression in the PFC \((r = -0.519, P=0.016 \text{ Fig. 3.17C})\). There were no further significant correlations between cytokine expression in any of the brain regions and behavioural responding post SNL.

**Fig. 3.17** Correlation between responding to acetone (cold) and neuroinflammatory mediators in the amygdala and PFC
3.3.11 Changes in brain monoamine levels following OB and SNL

Levels of serotonin (5HT), noradrenaline, dopamine and their metabolites in brain regions that modulate both mood and pain processing were assessed due to their role in affective and nociceptive processing (thalamus, amygdala, hippocampus, PFC).

3.3.11 Effect of OB and/or SNL on the levels of monoamines in the thalamus

Two-way ANOVA revealed an effect of SNL on noradrenaline levels in the thalamus ($F_{(1,32)} = 31.17, P<0.001$). *Post-hoc* analysis revealed that SNL increased noradrenaline levels in sham and OB animals when compared to NSNL controls (Fig 3.18A). Two-way ANOVA revealed an effect of OB on dopamine levels in the thalamus ($F_{(1,28)} = 5.81, P=0.023$), however *post-hoc* tests failed to reveal significant differences between groups. There was no effect of OB or SNL on levels of serotonin or its metabolite in the thalamus.
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Thalamus

A

**

## Conc. of Noradrenaline (ng/g of tissue)

Sham

OB

NSNL

SNL

B

## Conc. of 5HT (ng/g of tissue)

NSNL

SNL

C

## Conc. of 5HAA (ng/g of tissue)

NSNL

SNL

D

## Conc. of Dopamine (ng/g of tissue)

NSNL

SNL

E

## Conc. of DOPAC (ng/g of tissue)

NSNL

SNL

Fig. 3.18 Levels of (A) noradrenaline, (B) 5HT, (C) 5HAA, (D) Dopamine and (E) DOPAC in the thalamus. Data expressed as mean ± SEM. N=7-10. **p<0.01 vs. Sham-NSNL, #p<0.01 vs. OB-NSNL.
3.3.12 Effect of OB and/or SNL on the levels of monoamines in the amygdala

There was no significant effect of SNL on the levels of any of the monoamines or their metabolites in the amygdala. Although ANOVA revealed a significant effect of OB on 5HT levels ($F_{(1,31)} = 4.6$ $P=0.043$, Fig. 3.19C), post-hoc analysis failed to identify any significant differences in 5HT between animals.

**Amygdala**

Fig. 3.19 Levels of (A) noradrenaline, (B) 5HT, (D) 5HIAA, (D) Dopamine and (D) DOPAC in the amygdala. Data expressed as mean ± SEM. N=7-10
3.3.13 Effect of OB and/or SNL on the levels of monoamines in the hippocampus

Two-way ANOVA revealed an effect of SNL on levels of serotonin (5HT, $F_{(1,31)} = 7.77$, $P=0.009$) and its metabolite 5HIAA ($F_{(1,32)} = 12.28$, $P=0.001$) in the hippocampus. Post-hoc comparisons revealed that OB-SNL animals exhibited a decrease in hippocampal levels of serotonin and 5HIAA, when compared to NSNL counterparts (Fig 3.20 B&C), an effect not seen in sham animals. DOPAC and dopamine were below the limit of detection in this area.

Fig. 3.20: Levels of (A) noradrenaline, (B) 5HT, (D) 5HIAA in the hippocampus. Data expressed as mean ± SEM. $N=7-10$. *$P<0.05$, **$P<0.01$ vs. OB-NSNL.
3.3.14 Effect of OB and/or SNL on the levels of monoamines in the PFC

Two-way ANOVA revealed an effect of OB ($F_{(1,30)} = 6.07, P=0.02$), SNL ($F_{(1,30)} = 7.79, P=0.009$) and an OB × SNL interaction ($F_{(1,30)} = 24.59, P<0.001$) on dopamine levels in the PFC. Post-hoc comparisons revealed that OB rats exhibited lower levels of dopamine, but not any of the other monoamines, in the PFC compared to sham-operated counterparts (Fig. 3.21D). In addition, SNL resulted in decreased dopamine levels in the PFC of sham animals when compared to NSNL counterparts (Sham-SNL vs. Sham-NSNL, Fig. 3.21D). Although OB-SNL animals exhibited low levels of dopamine in the PFC, these levels did not significantly differ from either OB-NSNL or sham-SNL levels.

**Fig. 3.21** Levels of (A) noradrenaline, (B) 5HT, (D) 5HIAA, (D) Dopamine and (D) DOPAC in the PFC. **P<0.01 vs. sham-NSNL. Data expressed as mean ± SEM, n=7-11.**
3.4 Discussion

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**Neuroinflammatory mediators**

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<th>↑ IL-10</th>
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<th>↓ CD40, CD11b, GFAP, TNFα, ↑IL-6 (vs. OB-NSNL)</th>
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<td>↓ IL-6</td>
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**Monoamines**

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Table 3.2. Summary of behavioural, neuroimmune and monoaminergic changes from Chapter 3. The arrows indicate the direction of the changes; square brackets indicate the trends that were not statistically significant.
The present study characterised nociceptive responding in the OB rat model of depression, prior to and following SNL. In brief, these data demonstrate that the OB model of depression exhibits mechanical and cold allodynia in the absence of peripheral nerve injury. SNL induced characteristic mechanical and cold allodynia of the ipsilateral hind-paw of sham animals. OB-SNL animals exhibited mechanical allodynia of both the ipsi- and contra-lateral hind-paw and enhanced cold alldynia. Mechanical allodynia following SNL was inversely correlated with astrocyte activation in the dorsal horn of the spinal cord. The expression of inflammatory mediators in discrete brain regions responsible for the processing of emotion and pain was significantly altered in OB-SNL animals when compared to either OB or SNL alone, and OB-SNL animals demonstrated reduced serotonergic neurotransmitters in the hippocampus. Cold alldynia following SNL was positively correlated with IL-6 and IL-10 mRNA expression in the amygdala and negatively correlated with IL-10 expression in the prefrontal cortex. Finally, SNL increased serum corticosterone levels, increased noradrenaline levels in the thalamus and reduced dopamine levels in the PFC, effects not altered in OB animals. This study provides further evidence for altered pain responding in a model of depression and indicates that neuroimmune processes and brain monoaminergic neurotransmission may, in part, underlie the behavioural changes observed.

3.4.1 Altered affective behaviour in the OB model of depression

The current study confirmed the development of OB-induced depressive-like phenotype as indicated by increased locomotor activity on exposure to the open field test 2 weeks following OB surgery, an effect maintained following SNL. In addition, OB rats exhibited increased defecation and a tendency to spend less time in the inner zone, both measures of anxiety-related behaviour. Several studies from our own group and others have demonstrated similar effects of OB (Mar et al., 2002, Roche et al., 2012, Burke et al., 2010, Roche et al., 2008), though this is the first time that such effects have been examined following peripheral nerve injury, and indicates that this procedure does not alter depressive- or anxiety-like behaviour in these animals. However, as OB animals spent little time in the inner zone, it
may have been difficult for SNL to further reduce this measure. It should be noted that SNL resulted in a decrease in time in the inner zone of the open field in sham animals, indicative of anxiety-like behaviour, in accordance with previous studies (Suzuki et al., 2007, Hasnie et al., 2007a). Affective pain behaviour was examined in the PEAP, which is assumed to cause avoidance of noxious stimulation in the dark chamber, causing a shift towards the aversive light chamber (LaBuda and Fuchs, 2000a). In contrast to previous findings (LaBuda and Fuchs, 2000a, 2005, 2000b), our study demonstrated that SNL rats spent less time in the light compartment when compared to non-SNL rats (sham-operated). Methodological differences such as high light levels used (200 lux vs. ambient), repeated von Frey testing prior to the PEAP, or time of testing relative to NSNL/SNL surgery (day 21 vs. day 3-4) may account for the discrepancy between studies. A recent study reported that 7 days post-nerve injury, CCI rats exhibited increased time in the light chamber, indicating enhanced affective pain behaviour (Alba-Delgado et al., 2013). However, after 28 days of neuropathy, rats spent less time in the light, despite a similar level of mechanical allodynia. This was accompanied by increased anxiety-like and depressive-like behaviour in the elevated zero maze and forced swim test, respectively. Thus, it appears that long-term SNL results in anxiety-like behaviour in both the open field (14 days) and PEAP arena (21 days). In addition, the data demonstrated that OB animals spent little time in the light-side of the chamber, regardless of SNL. The profile of reduced time spent in the light side of the PEAP also mirrors the reduced time spent in the inner zone of the open field following SNL, and correlates with reports of increased OB-induced anxiogenic behaviour in the open field, elevated plus maze and social interaction tests (Hallam et al., 2004, Zueger et al., 2005, Wang et al., 2007), lending further evidence for OB-induced anxiety-related behaviour. Further, as OB rats display bilateral mechanical allodynia prior to and following SNL, stimulation of either paw is likely to induce an allodynic response. Therefore, the aversive stimulation would be assumed to be equal in the dark and the light, and the conflict between sides would not have arisen. Thus, as bullectomised rats exhibit bilateral allodynia and appear to exhibit anxiogenic behaviour in the PEAP, strong conclusions
regarding affective pain behaviour in OB animals are evaded. Taken together, these data indicate depressive- and anxiety-like behaviour in the OB rat, an effect not altered by peripheral nerve injury.

3.4.2 Altered nociceptive responding in the OB model of depression

The present study demonstrated that OB rats exhibit mechanical allodynia, exemplified as increased sensitivity to von Frey filaments, results consistent with those previously reported from our laboratory (Burke et al., 2010). Previous studies assessing mechanical hyperalgesia using the paw pressure test have reported both decreased (Belcheva et al., 2009) and increased (Rodríguez-Gaztelumendi et al., 2006) sensitivity in OB rats. In the current study, nociceptive responding to thermal stimuli (heat and cold) revealed that OB rats exhibit increased sensitivity (i.e. reduced latency to respond) to cold (acetone) but not heat stimuli (Hargreaves test). In comparison, previous data from our group have demonstrated that OB rats exhibit transient heat hyperalgesia in the hot plate, but not tail flick, test (Burke et al., 2010), and data from other groups have reported increased paw withdrawal latency to a radiant heat source in OB rats (Su et al., 2010, Wang et al., 2010b). Thus, depending on the test employed, OB animals appear to exhibit slight alterations in nociceptive responding to thermal stimuli. Taken together, these data correlate with those observed clinically where depressed patients exhibit decreased (Bar et al., 2007, Schwier et al., 2010, Lautenbacher et al., 1994), increased (Strigo et al., 2008b, Chiu et al., 2005, Gormsen et al., 2004) and no change (Graff-Guerrero et al., 2008) in sensitivity to experimental pain, depending on the modality and intensity of the stimulus.

The current study is the first to examine the effect of OB on nociceptive responding following spinal nerve ligation, a clinically-relevant model of persistent neuropathic pain. These data demonstrated that OB rats exhibited ipsi- and contra-lateral mechanical allodynia and enhanced cold allodynia following SNL. Although SNL has been reported to induce contralateral alldynia, an effect which develops later and is of less intensity than allodynia of the ipsilateral hind-paw (Arguis et al., 2008), such an effect was not observed in sham animals in the current study. However, OB-SNL
animals exhibited lower contralateral mechanical thresholds compared to sham-SNL animals. As OB animals exhibited lower thresholds prior to SNL, this may explain the contralateral hypersensitivity following SNL. Although not observed in the current study, enhanced mechanical allodynia following CCI and spared nerve injury has been reported in other animal models of depression, including the WKY rat and chronic restraint stress (Zeng et al., 2008a, Norman et al., 2010b). In comparison, increased mechanical and thermal thresholds have been reported following SNL and partial sciatic nerve ligation in the unpredictable chronic mild stress (UCMS) (Shi et al., 2010a) and the Flinders sensitive line (Shir et al., 2001) models of depression, respectively. UCMS and OB have strikingly divergent effects on nociceptive responding following SNL, which may be accounted for by differences in experimental design. For example, UCMS began 7 days following SNL, whereas in our study SNL began 19 days after OB surgery. It must be noted that these differences may also be related the nature of the models (stress vs. lesion), the neurobiological mechanisms underpinning the pathogenesis of these models, and the subsequent impact upon pain pathways.

3.4.3 SNL increases serum corticosterone levels, an effect not altered in OB animals

Numerous studies have investigated corticosterone levels in the OB model demonstrating increased (Rinwa et al., 2013, Song et al., 2009, Marcilhac et al., 1999, Cattarelli and Demael, 1986), reduced (Song and Leonard, 1995) and no change (Williams et al., 1992, Cairncross et al., 1977, Broekkamp et al., 1986, Breivik et al., 2006b) in basal levels. Broekkamp et al suggested that OB animals exhibit increased stress-induced corticosterone response as opposed to increased basal levels (Broekkamp et al., 1986). Furthermore, corticosterone secretion is reportedly enhanced only in the dark phase of the circadian cycle (Kelly et al., 1997, Song et al., 1994). The lack of OB-related alterations in serum corticosterone levels may not be surprising as samples were collected during the light-phase at 24 hours following behavioural testing when stress responses would have returned to baseline.
Despite the potential role of the HPA axis in chronic pain, there are a lack of studies examining interactions between this system and neuropathic pain (Blackburn-Munro and Blackburn-Munro, 2001). Most studies to date report no alterations in basal corticosterone levels after SNI (Alexander et al., 2009, Wang et al., 2011), CCI (Bomholt et al., 2005b), or sciatic nerve cuffing (Yalcin et al., 2011). Nonetheless, increased expression of CRF and GR mRNA in the amygdala following CCI has been reported (Ulrich-Lai et al., 2006). In accordance with the current data where SNL increased serum corticosterone levels in both sham and OB rats, it has been reported that plasma corticosterone is increased in CCI animals 1-14 days post-surgery (Wang et al., 2004), suggesting that chronic pain elicits activation of the HPA axis, an effect also seen clinically (Van Uum et al., 2008). Peripheral nerve injury results in noradrenergic impairment in the locus coeruleus (Alba-Delgado et al., 2013) which may elicit activation of the HPA axis via CRF (Dunn and Swiergiel, 2008). These data suggest that alterations in corticosterone levels do not account for the differential nociceptive responding or neuroimmune alterations observed between sham and OB rats after SNL.

### 3.4.4 Neuroimmune changes associated with altered nociceptive responding in the OB model

The present study demonstrated that SNL resulted in microglial and astrocyte activation in the ipsilateral L5 dorsal horn of the spinal cord, correlating with previous studies (Wang et al., 2008b, Jin et al., 2003). A wealth of evidence now suggests that spinal microglia are involved in the development of neuropathic pain, while in comparison, astrocytes have been implicated in its maintenance (Raghavendra et al., 2003, Kronfol and Remick, 2000, Colburn et al., 1999, Winkelstein and DeLeo, 2002, Narita et al., 2006c). Although SNL did not alter OX42 and GFAP immunostaining in the contralateral dorsal horn of sham animals, OB animals displayed increased astrocyte activation in both the ipsi- and contra-lateral dorsal horn following SNL. Correlation analysis revealed that GFAP immunoreactivity in the spinal cord was inversely correlated with mechanical thresholds
following SNL, indicating that astrocyte activation may modulate nociceptive responding to mechanical stimuli following nerve injury. Spinal astrocytes have been implicated in the development of mirror-image mechanical allodynia following nerve injury (Obata et al., 2010), an effect which may account for the contralateral mechanical allodynia observed in OB rats following SNL surgery. While OB animals exhibited lower thresholds of the contralateral hind-paw prior to SNL, enhanced astrocyte activation in the contralateral dorsal horn of OB-SNL animals was not observed in non-SNL OB animals. Although the exact means by which bulbectomy may influence spinal astrocyte activation remains to be determined, it is possible that enhanced circulating pro-inflammatory cytokines and prostaglandins may play a role (Song et al., 2009) or neuronal reorganisation within limbic and cortical areas following bulbectomy may modulate the descending pain pathways and ultimately spinal processes.

As highlighted earlier, there is increasing evidence to suggest that inflammatory mediators in discrete brain regions modulate both affective and nociceptive processing, and accordingly may underlie the altered nociceptive responding associated with depression. The thalamus is an important relay site for both the affective and sensory/discriminative aspects of pain. Recent studies have demonstrated that microglial activation in the thalamus is associated with thermal allodynia following CCI and in streptozotocin-induced diabetic mice (LeBlanc et al., 2011, Toth et al., 2010). To our knowledge, the current study is the first to examine glial and cytokine expression in this brain region following SNL in rats. Our data suggest that SNL per se is associated with minimal alterations in the expression of pro-inflammatory immune mediators at the level of the thalamus, although expression of the anti-inflammatory cytokine IL-10 was increased in this brain region by SNL. The relative lack of change in pro-inflammatory cytokine expression following SNL may be unsurprising given the time at which these were examined post surgery (day 22). Previous studies have shown that GFAP, IL-1β, TNFα and IL-6 protein levels are increased in the brain between day 3-7 after peripheral nerve injury (Liu et al., 2007, Xie et al., 2006, Marcello et al., 2013), correlating
with the development and peak of neuropathic pain behaviours in the model. In comparison, IL-10 levels in the brain increase over time following SNL, peaking 21 days post surgery (Xie et al., 2006), thus correlating with the present findings. In comparison, OB rats exhibited increased thalamic IL-6 expression with a concurrent reduction in the expression of TNFα and markers of microglial and astrocyte activation following SNL. Furthermore, OB animals exhibit a significant increase in CD11b, with a similar trend for CD40 and GFAP, an effect not observed following SNL. Thus, SNL induces differential effects on the expression of inflammatory mediators in the thalamus in sham and OB animals.

The PFC and limbic regions such as the amygdala and hippocampus play central roles in the processing of emotion and pain. OB rats have been shown to exhibit increases in TNFα and IL-1β levels in the PFC, hippocampus and hypothalamus (Myint et al., 2007), increased phospholipase A2 and prostaglandin E2 in the hypothalamus (Song et al., 2009) and increased GFAP in the frontal cortex (Cizkova et al., 1997). In contrast with these earlier findings, there was no effect of bulbectomy on microglial or astrocyte activation or cytokine expression in the PFC or hippocampus in the current study. Methodological differences such as time post-OB surgery and effect of NSNL surgery may account for the discrepancy. For example, Borre et al. (2012b) reported increased microglial activation on day 3 and increased IL-1β expression on day 7 in the hippocampus post-bulbectomy, effects which returned to control levels by day 28. However, in the current study, increased microglial and astrocyte activation and enhanced IL-1β expression was observed in the amygdala of OB rats 41 days post-bulbectomy. To our knowledge, this is the first study to investigate the effect of bulbectomy on immune mediators in the amygdala, although OB-induced changes in neuronal activation, monoamines and neuropeptides have been observed in this brain region (Roche et al., 2012, Roche et al., 2007, van der Stelt et al., 2005, Rutkoski et al., 2002). OB-induced hyperactivity is associated with neurodegeneration in the amygdala (Wrynn et al., 2000b, Jarosik et al., 2007), an effect which may result from increased neuroinflammation in this region following
removal of the bulbs. Increased glial activation and IL-1β within the amygdala may be responsible for OB-induced mechanical and cold allodynia, as it has been shown that intracerebroventricular administration of non-pyrogenic doses of IL-1β results in thermal (Oka et al., 1993) and mechanical (Yabuuchi et al., 1996) hyperalgesia. Interestingly, the increase in the expression of IL-1β in the amygdala of OB rats was not observed following SNL, suggesting that peripheral nerve injury can attenuate the increases in amygdaloid IL-1β expression that result from central injury (OB). CCI has been shown to decrease NFκB expression in the amygdala (Chou et al., 2011b), and thus reduced NFκB may inhibit transcription of cytokine genes. Indeed, SNL induced a decrease in IL-6 expression in the amygdala, an effect further enhanced in OB rats, and increased IL-10 expression in sham, but not OB animals. Paw withdrawal latency to a cold stimulus was positivity correlated with IL-6 expression in the amygdala, thus enhanced initial cold perception observed in OB animals is associated with, and possibly mediated by, low IL-6 levels in the amygdala. IL-6 suppresses glutamate release in the cortex (D'Arcangelo et al., 2000), reduces NMDA-induced Ca$^{2+}$ overload (Sun et al., 2011), and protects against NMDA-induced neuronal damage (Liu et al., 2011b). Glutamate receptors in the amygdala have been shown to contribute to the maintenance of sensory and emotional components of neuropathic pain (Ansah et al., 2010). Thus, although hypothetical, it is possible that decreased IL-6 in the amygdala may result in disinhibition of NMDA receptors and consequently facilitation of thermal nociceptive processing. Furthermore, bulbectomy is associated with reduced NMDA receptor density but enhanced activity in the amygdala (Ho et al., 2001, Nakanishi et al., 1990), which may account for the enhanced cold alldynia observed in OB animals post-SNL. Normalised withdrawal frequency to an innocuous cold stimulus was positively correlated with IL-10 expression in the amygdala. As mentioned, IL-10 levels in the brain have been reported to be enhanced following SNL (Xie et al., 2006), although region-specific changes have not been investigated. Pharmacological and genetic deletion of IL-10 is associated with depressive-like behaviour (Mesquita et al., 2008) and increased thermal nociceptive thresholds (Tu et al., 2003). IL-10 is known to protect against
glial and neuronal cell death (Strle et al., 2002, Bachis et al., 2001) and thus changes in IL-10 expression may alter neuronal integrity and function with the amygdala. In the PFC, the expression of markers of microglial and astrocyte activation, and IL-1β and TNFα were increased in OB animals following SNL. Indeed, IL-1β expression in the frontal cortex has been shown to be increased in other models of depression following nerve injury (Apkarian et al., 2006, Norman et al., 2010a, Norman et al., 2010b), and antagonism of IL-1 has been shown to attenuate the effects of neuropathic pain on depressive behaviour (Norman et al., 2010b). The functional role of inflammatory mediators in the PFC on affective and nociceptive behaviour in the present model remains to be determined, yet these studies and data presented in this chapter indicate that the combination of depression and pain leads to enhanced inflammatory gene expression in this key brain region.

3.4.5 Monoamine levels in the brain are differentially altered by OB and SNL

The monoamines are well-known mediators in the modulation of mood and nociceptive processes, and it has been shown that monoamine depletion (reserpine) results both depressive-like behaviour and mechanical allodynia in rats (Arora et al., 2011, Nagakura et al., 2009) and antidepressant drugs that increase monoaminergic neurotransmission have been shown to be effective in reducing OB-associated behavioural deficits (King and Cairncross, 1974, Redmond et al., 1999, McGrath and Norman, 1998, Jarosik et al., 2007, Roche et al., 2008). As discussed, the OB rat has been proposed as a model of hyposerotonergic depression (Lumia et al., 1992, Redmond et al., 1997, Connor et al., 1999, van der Stelt et al., 2005, Hellweg et al., 2007). However, in the current study, we did not find reductions in 5HT levels or its metabolite 5HIAA in discrete brain regions of OB animals, in accordance with previous reports from our laboratory (Burke et al., 2010). Components of the experimental design, such as NSNL surgery or the time-point at which these monoamines were examined, may account for the lack of serotonergic deficits observed here. We did note
reduced dopamine levels in the PFC of OB animals, an effect corroborating previous reports (Jesberger and Richardson, 1988).

In the current study, SNL resulted in increased noradrenaline levels in the thalamus and decreased dopamine levels in the PFC. To our knowledge, characterisation of supraspinal levels of monoamines following SNL has not yet been carried out, thus the current data is the first to describe these changes. However, other models of neuropathic pain have reported that serotonin levels are decreased in pain-processing regions including the cerebral cortex, the ventrobasal thalamus, the raphe magnus nucleus, and the spinal cord (Goettl et al., 2002, Hains et al., 2001, Liu et al., 2010, Sandrini et al., 1997, Sounvoravong et al., 2004). Chronic constriction injury has been shown to enhance noradrenaline signalling in the locus coeruleus (Alba-Delgado et al., 2013), the primary cell body area for noradrenaline supply to the thalamus and other forebrain regions. It is possible that a similar effect occurs following SNL leading to selective increases in noradrenaline levels in discrete brain regions. Decrements in PFC dopamine levels have been associated with inflammatory pain-related cognitive impairment (Pais-Vieira et al., 2009) and anxiety-like behaviour (Espejo, 1997). Thus, reduced dopamine in the PFC may account for the increased anxiety-like behaviour observed in the open field and PEAP in OB and SNL animals.

The combination of OB and SNL resulted in reduced concentration of 5-HT and 5HIAA in the hippocampus compared to NSNL controls. Bulbectomy alone reportedly decreases serotonin turnover in the hippocampus (Hellweg et al., 2007), and SNL alone has been reported to reduce the density 5-HT transporter in the hippocampus (Rojo et al., 2012). Although reduced serotonin was not observed with OB or SNL alone at the current time-point, it is possible that the combination of OB and SNL resulted in a more prolonged reduction of this monoamine in the hippocampus. We previously reported that OB rats demonstrate a blunted formalin-induced increase in 5HIAA in the hippocampus (Burke et al., 2010). Taken together, these data suggest that deficient serotonergic neurotransmission in the hippocampus may be implicated in altered nociceptive responding in the OB model.
3.5 Conclusion

In conclusion, the data presented herein characterised nociceptive responding in the OB model of depression prior to and following peripheral nerve injury, demonstrating bilateral mechanical allodynia and enhanced cold allodynia following nerve injury. These data provide evidence that behavioural changes are accompanied by alterations in monoaminergic and inflammatory mediators in discrete brain regions involved in nociception and emotion. The results presented in this chapter provide a basis for further investigation of the neural substrates mediating altered nociceptive responding in this model.

3.6 Summary of aims and subsequent findings

(1) Characterised nociceptive responding to mechanical, heat and cold stimuli prior to and following peripheral nerve injury in the OB rat

(i) OB exhibit mechanical and cold alldynia prior to nerve injury, and exacerbated cold, but not mechanical alldynia following SNL.

(2) Investigated if affective pain-related behaviour following SNL is altered in OB

(ii) Not concluded – confounded by potential anxiogenic-like behaviour

(3) Investigated if OB-related hyperactivity in open field persists following SNL

(iii) Yes.

(4) Examine if SNL-induced glial activation at the level of the spinal cord is altered in the OB model of depression.

(iv) OB exhibit bilateral astrocyte activation following SNL.

(5) If serum corticosterone levels are altered by OB, SNL and their interaction.

(v) Enhanced by SNL, but not OB.

(6) Determine if interactions between OB and SNL at a behavioural level are accompanied by alterations at the neuroimmune and monoaminergic level in discrete brain regions responsible for the processing of emotion and pain (PFC, amygdala, thalamus and hippocampus).

(vi) Yes, a number of region-dependent alterations.
Chapter 4

The effect of chronic amitriptyline treatment on depressive-like behaviour and nociceptive responding prior to and following peripheral nerve injury in the OB rat: associated alterations in inflammatory mediators in the prefrontal cortex and amygdala
4.1 Introduction

Compelling evidence for shared substrates in depression and pain arises from the efficacy of antidepressant pharmacotherapy in the treatment of chronic pain. The analgesic and antidepressant effects of these agents are considered to occur independently (Mico et al., 2006). That is, antidepressants have analgesic effects in chronic pain patients without depression, and analgesic efficacy can occur without any effect on mood in depressed patients with comorbid pain (Saarto and Wiffen, 2010). Nonetheless, in certain cases, effects of antidepressant treatment on mood improvement have been shown to account for a portion of the pain relief observed in depressed patients (30-50%) (Marangell et al., 2011, Perahia et al., 2006). However, the mechanisms by which antidepressants alleviate pain in the presence or absence of depression remain to be elucidated.

Neuropathic pain remains one of the most complex and difficult chronic pain conditions to treat. Newer non-antidepressant treatments used for neuropathic pain include anticonvulsants, neuromodulation, and topical and transdermal preparations (Freeman, 2005). However, some of these treatments only reduce pain severity by 30-40% (Turk, 2002) and do not have the added benefit of alleviating depression (Dworkin et al., 2007). Amitriptyline (AMI) is a tricyclic antidepressant which inhibits the re-uptake of noradrenaline and serotonin and remains a first-line treatment for neuropathic pain (Finnerup et al., 2005) and is considered the gold-standard of analgesic antidepressants (Mico et al., 2006). In recent years, selective serotonin reuptake inhibitors (SSRI’s) are used more frequently for the treatment of depression than the older tricyclic antidepressants (TCA’s). However, SSRI medications do not possess substantial analgesic properties (Coluzzi and Mattia, 2005), and dual re-uptake inhibition is more efficacious in the treatment of chronic pain (Mico et al., 2006). Although the presence of depression is not required for the analgesic effects of TCA’s, these antidepressants may be especially useful for alleviating neuropathic pain in patients with inadequately treated depression (Dworkin et al., 2007).
Indeed, it has been shown that AMI is more effective than other non-monoaminergic drugs (diphenhydramine, gabapentin) for the treatment of neuropathic pain in patients with high depressive symptoms compared to those with low depressive symptoms (Rintala et al., 2007). As such, tricyclic antidepressants or selective serotonin–noradrenaline reuptake inhibitors (SNRI’s, e.g. duloxetine, venlafaxine) may be a preferred treatment for patients with depression-pain co-morbidity (Gupta et al., 2007, Bradley et al., 2003, Brecht et al., 2007, Ward et al., 1984, Ward et al., 1979, Sullivan et al., 1992).

The mechanisms of action by which AMI exerts its antinociceptive effects are not yet fully understood, but it is known to work at a variety of central and peripheral targets. AMI may work by facilitating the descending inhibition of pain by increasing central monoamine levels (Beydoun and Backonja, 2003), by binding to opioid, adenosine, histamine and acetylcholine receptors (Esser and Sawynok, 2000, Valverde et al., 1994) and/or binding at peripheral sodium, potassium and calcium channels (Sindrup et al., 2005). This varied pharmacology of AMI means that the high doses required for the treatment of depression with this agent can result in a wide range of side effects which may limit patient compliance. However, the treatment of neuropathic pain with AMI has been reported to require lower doses and a shorter treatment duration than that required to treat depression (McQuay et al., 1996), thus side effects are generally limited and adherence improved.

Relatively few animal studies have investigated the effect of antidepressants on the interaction between depression and pain. Clomipramine, a tricyclic antidepressant, alleviates depressive- and anxiety-like behaviours and reverses mechanical, but not thermal hypoalgesia, in the learned helplessness model of depression (Li et al., 2013). In the CCI and L5 spinal nerve transection models of peripheral nerve injury, acute (Jesse et al., 2010) and chronic (Hu et al., 2010) AMI treatment ameliorates both mechanical allodynia and depressive-like behaviour in the forced swim test. Furthermore, chronic AMI has been demonstrated to attenuate OB-
Chapter 4 – Effect of amitriptyline in OB-SNL

associated enhanced nociceptive responding in a model of trigeminovascular nociception (Liang et al., 2011). Data presented in Chapter 3 demonstrated that the OB rat exhibits mechanical and cold allodynia prior to nerve injury, and exacerbated cold allodynia following SNL. Previous data have demonstrated that chronic AMI treatment reverses OB-induced hyperactivity and passive avoidance deficits (van Riezen et al., 1977, Mar et al., 2000), and attenuates SNL-induced thermal hyperalgesia (Cheng et al., 2012, Esser et al., 2001). Therefore, the aim of this study was to investigate the effect of chronic AMI administration on nociceptive and affective pain responding in the OB model of depression, prior to and following peripheral nerve injury (SNL).

The behavioural changes observed in Chapter 3 were associated with alterations in the expression of inflammatory markers and monoamine levels in discrete brain regions. Specifically, the combination of OB and SNL resulted in enhanced expression of inflammatory mediators in the PFC and SNL-induced cold allodynia was correlated with inflammatory mediator expression in the amygdala. Thus, these two regions were chosen for further study. As AMI increases monoamine levels in the brain, we examined the effect of chronic AMI treatment on the levels of monoamines in the PFC and amygdala following OB and SNL.

Further evidence supporting a role for inflammation in the link between depression and pain arises from anti-inflammatory effects of antidepressants, as in addition to modulation of monoaminergic neurotransmission, antidepressants have been shown to possess immunomodulatory properties (Janssen et al., 2010, Castanon et al., 2002, Diamond et al., 2006, Basterzi et al., 2005). AMI has been found to decrease the levels of IL-1β and TNFα released by mixed glial cell cultures (Obuchowicz et al., 2006) and increase secretion of IL-10 from mouse splenocytes (Kubera et al., 2000). AMI attenuates the pro-inflammatory effect of repeated morphine treatment in the spinal cord (Tai et al., 2009, Tai et al., 2006). The TCA desipramine reduces LPS-stimulated expression of IL-1β, TNFα, iNOS, CD11b and CD40 in the cortex (O'Sullivan et al., 2009) and in OB rats, chronic desipramine treatment decreased LPS-
induced release of TNFα and IL-1β in the plasma (Connor et al., 2000). Acute AMI inhibits iNOS mRNA and protein expression in the cerebellum and hippocampus following chronic constriction injury (Farghaly et al., 2012), while pre-surgical AMI attenuates SNL-induced activation of astrocytes in the spinal cord (Cheng et al., 2012). However, it is not known whether chronic AMI treatment alters the mRNA expression of neuroinflammatory mediators in supraspinal regions differentially in sham and OB rats following SNL. Therefore, a further aim of the current study was to examine if chronic AMI treatment modulates the expression of inflammatory mediators in the PFC and amygdala of sham and OB animals following peripheral nerve injury.

4.1.2 Hypothesis and aims

Chronic amitriptyline treatment would elicit an antidepressant-like effect in the OB and differentially alter nociceptive responding, prior to and following SNL, effects accompanied by altered expression of neuroimmune mediators and/or monoamine levels in the PFC and amygdala

1. Examine if chronic AMI treatment alters depressive-like behaviour in the OB rat
2. Determine if chronic AMI alters nociceptive responding to mechanical and thermal stimuli, and if such an effect is altered in OB animals
3. Determine the effect of chronic AMI on SNL-induced mechanical and cold allodynia (sham animals)
4. Investigate if chronic AMI induces differential effects on SNL-induced allodynia in OB rats
5. Determine if chronic AMI treatment alters affective pain behaviour following SNL, and if this differs in OB animals
6. Evaluate the effect of chronic AMI on levels of monoamines and inflammatory mediators in discrete brain regions associated with affective and nociceptive processing (PFC and amygdala)
4.2 Materials & Methods

4.2.1 Animal Husbandry

Adult male Sprague Dawley rats weighing 180-220g on arrival were obtained from Charles River (Margate, UK). Animals were singly housed under standard conditions as described in Chapter 2 (Section 2.1, Materials and Methods), and food and water were available ad libitum. The experimental protocol was carried out following approval from the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Department of Health and Children in the Republic of Ireland and in accordance with EU Directive 86/609.

4.2.2 Drug administration

Amitriptyline hydrochloride (Sigma, Ireland) was administered intraperitoneally at a dose of 10mg/kg in an injection volume of 2ml/kg, dissolved in sterile saline (pH 7.4). Animals were injected daily beginning on the day of OB/sham surgery, at least 15 hours prior to behavioural testing. Vehicle-treated groups received sterile saline injections. The choice of dose was based on the antidepressant effects of chronic AMI in OB rats (van Riezen et al., 1977), and the anti-nociceptive effects following L5/L6 SNL (Esser et al., 2001).

4.2.3 Experimental design

The experimental design is presented in Figure 4.1 and tests were carried out as described in Chapter 2, Materials and Methods. Essentially, male Sprague Dawley rats were tested during the light phase in the open field (locomotor activity, section 2.4.1.1), von Frey (mechanical, section 2.4.2.1), acetone-drop test (cold, section 2.4.2.3) at baseline to ensure no differences between groups assigned to sham or OB surgery. The Hargreaves test (heat, section 2.4.2.2) was not carried out prior to surgery due to concerns about repeated testing in this behavioural assessment (Kocevski and Tvrdeic, 2008). Animals were randomly assigned to either sham (n=24) or olfactory bulbectomy (OB) (n=25), and surgery was then performed as described in Chapter 2 (Section 2.2). Animals were further divided randomly into four
treatment groups as follows: Sham-Vehicle (Sham-Veh, n=12), Sham-amitriptyline (Sham-AMI, n=12), olfactory bulbectomy-Vehicle (OB-Veh, n=13) and olfactory-bulbectomy-amitriptyline (OB-AMI, n=12). Amitriptyline (AMI), or vehicle was administered daily beginning on the day of OB/sham surgery. Animals were allowed two weeks to recover before behavioural testing for locomotor activity and nociceptive responding to mechanical and thermal stimuli (including Hargreaves test, heat). Twenty-four days post-bulbectomy, L5/L6 Spinal Nerve Ligation (SNL) surgery was carried out on all animals, as described in Chapter 2 (Section 2.3). Mechanical withdrawal thresholds were examined on days 3, 7, 10 and 14 post-SNL. Animals were also tested in the acetone-drop test (Day 14), Hargreaves (Day 17) and PEAP (Day 18, section 2.4.2.4). Animals were sacrificed by rapid decapitation 24 hours after the last behavioural test, and at least 15 hours after the final AMI dose. The brain was removed and the PFC and amygdala dissected out on an ice-cold plate, separated into right and left, and stored at -80\(^\circ\)C until assessed for mRNA expression of inflammatory markers (Section 2.7) and monoamine content (Section 2.8) as described in Chapter 2.

Fig. 4.1 Experimental timeline. AMI – Amitriptyline; AT – acetone drop test, HG – Hargreaves; OB – olfactory bulbectomy; OF – open field; PEAP – place escape/avoidance paradigm; SNL – spinal nerve ligation; VF – von Frey test
4.2.4 Statistical analysis

PASW 18 statistical program was used to analyse all data. All data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene’s tests respectively. The statistical significance of differences between groups was assessed using t-tests or two-way analysis of variance (ANOVA), using factors of OB or amitriptyline treatment, or repeated measures ANOVA using factors of time, followed by Fisher’s least significant difference (LSD) post-hoc testing where appropriate. Non-parametric data were assessed using Kruskal-Wallis to determine overall differences between groups, Friedman’s test to assess changes over time, and Mann-Whitney U post-hoc tests where appropriate. Pearson’s (for parametric data) and Spearman’s (for non-parametric data) analysis was carried out to examine correlations between behaviour and inflammatory mediator expression in the brain. The level of significance was set at P<0.05. All data are presented as the mean ± SEM.
4.3 Results

4.3.1 Locomotor activity and nociceptive responding prior to surgery

Behaviour in the open field or nociceptive responding did not differ between groups assigned to sham or OB prior to surgery (Table 4.1).

<table>
<thead>
<tr>
<th>Behavioural Test</th>
<th>Sham</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open field</strong></td>
<td>Distance moved (cm)</td>
<td>2449±79</td>
</tr>
<tr>
<td><strong>von Frey test</strong></td>
<td>Mechanical withdrawal thresholds (g)</td>
<td>5.8±0.5</td>
</tr>
<tr>
<td></td>
<td>Lowest filament at which response elicited (g)</td>
<td>6.2±1</td>
</tr>
<tr>
<td><strong>Acetone drop test</strong></td>
<td>Latency to respond (s)</td>
<td>37±3</td>
</tr>
<tr>
<td></td>
<td>Withdrawal frequency</td>
<td>0.8±0.2</td>
</tr>
</tbody>
</table>

Table 4.1. Baseline locomotor activity and nociceptive responding.
Data presented as mean ± SEM. n=24-25
4.3.2 Effect of bulbectomy and chronic AMI treatment on body weight

Body weight was measured daily to examine effects of bulbectomy and chronic drug treatment on weight gain over the study period. For clarity, data are presented and were analysed as the values on the day prior to OB surgery, 7 and 14 days post-sham/OB surgery, the day following SNL, and 7 and 14 days post-SNL. Repeated measures two-way ANOVA revealed effects of OB ($F_{(1,41)} = 34.55, P<0.001$), AMI ($F_{(1,41)} = 44.03, P<0.001$), time ($F_{(6,246)} = 338.90, P<0.001$), time × OB ($F_{(6,246)} = 19.48, P<0.001$), time × AMI ($F_{(6,246)} = 22.765, P<0.001$), time × OB × AMI interaction ($F_{(6,246)} = 3.224, P=0.005$) on body weight. Post-hoc analysis revealed that all animals gained weight over the study period. Body weight did not differ between groups prior to surgery, however weight of OB rats was significantly lower compared to sham-counterparts at all time-points examined post OB-surgery (Fig. 4.2). Sham-AMI treated animals exhibited lower body weight on day 7 post-OB and day 14 post-SNL surgery when compared to vehicle-treated sham-counterparts. In addition, OB-AMI animals displayed lower body weight when compared with vehicle-treated OB counterparts on day 14 post-OB until day 14 post-SNL inclusive.
Fig. 4.2 (A) Body weight (g) over the study period. **P<0.01 vs. Sham-Veh, ##P<0.01 vs. OB-Veh. Data expressed as mean ± SEM, n=12-13.
4.3.3 OB-induced hyperactivity is prevented by chronic amitriptyline treatment

Repeated measures two-way ANOVA revealed an effect of OB ($F_{(1,43)} = 4.76$, $P=0.035$), AMI ($F_{(1,43)} = 12.39$ $P=0.001$), time ($F_{(4,172)} = 14.69$ $P<0.001$), and a time × OB interaction ($F_{(4,172)} = 12.52$ $P<0.001$) on distance moved per minute over the 5 minute trial period in the open field 14 days post OB/sham surgery. Post-hoc analysis revealed that OB (vehicle-treated) rats displayed an increase in distance moved during the first 3 minutes of the test when compared to their sham counterparts (Fig. 4.3A). Chronic AMI treatment did not alter locomotor activity in sham animals (sham-AMI vs. sham-veh), however, AMI significantly prevented OB-induced hyperactivity at all time-points examined (OB-AMI vs. OB-veh, Fig 4.3A). Further analysis of total distance moved over the 5 min in the open field revealed an effect of OB ($F_{(1,43)} = 4.77$, $P=0.035$) and AMI ($F_{(1,43)} = 12.39$, $P=0.001$) (Two-way ANOVA). Post-hoc analysis revealed that OB animals demonstrated a characteristic increase in locomotor activity, compared to their sham counterparts, which was significantly attenuated by chronic AMI treatment (Fig. 4.3B). Anxiety-like behaviour was assessed by examining time spent in the inner zone of the open field, and two-way ANOVA revealed a significant effect of OB ($F_{(1,41)} = 8.324$, $P=0.006$). OB vehicle-treated animals spent less time in the inner zone when compared to their sham counterparts, indicative of an anxiogenic effect (Fig. 4.3C), and a similar effect was observed in AMI-treated OB animals, although this failed to reach statistical significance ($P=0.055$). Neither OB nor AMI altered faecal pellet count, rearing or grooming duration in the open field (Fig. 4.3 D-F).

Animals were re-exposed to the open field one week later (day 21 post OB-veh/AMI treatment). OB rats did not display the characteristic hyperactivity response when assessed at this time, possibly due to habituation and reduced novelty due to repeated exposure to this test. However, it should be noted that OB animals continued to display a decreased time in the inner zone of the open field (data presented in appendix, Fig. B.3).
Fig. 4.3 Behaviour in the open field two weeks following sham/OB surgery (A) Distance moved in the open field over 5 minute time bins (B) total distance moved (C) Time in the inner zone (D) Faecal pellet count in the open field (E) Rearing Duration (F) Grooming duration. *P<0.05, **P<0.01 vs. sham-veh, ##P<0.01 vs. OB-Veh. +P<0.05 vs. sham-AMI. Data expressed as mean ± SEM, n=12-13.
4.3.4 Effect of OB and/or AMI treatment on nociceptive responding to mechanical, cold or heat stimuli prior to nerve injury

The effect of bulbectomy and chronic AMI treatment on nociceptive responding to mechanical and thermal stimuli was examined. Neither OB nor AMI significantly altered 50% mechanical withdrawal thresholds of the hind-paws (Fig. 4.4A). Assessment of the lowest filament force to elicit a response using Kruskal-Wallis test revealed an overall effect of group ($\chi^2(3) = 10.13$ $P=0.037$, Fig. 4.4B). Mann-Whitney post-hoc tests revealed that OB animals responded at lower von Frey filaments when compared to sham controls, indicative of mechanical allodynia, with a trend in the same direction observed in OB AMI-treated animals. Chronic AMI treatment did not alter mechanical withdrawal thresholds in sham animals. Neither OB nor AMI altered paw withdrawal latency or frequency to respond to an innocuous cold stimulus, or withdrawal latency to a noxious heat stimulus (Fig. 4.4 B-E).
Fig. 4.4 Nociceptive responding to mechanical, cold and heat stimulation. (A) von Frey filament force eliciting a 50% response of the hind-paws following sham/OB surgery. (B) Lowest von Frey filament force to elicit a response of the hind-paws (C) Paw withdrawal latency and (D) withdrawal frequency to acetone application to the hind-paws. (E) Paw withdrawal latency to application of a noxious heat stimulus to the hind-paws. *P<0.05 vs. Sham-Veh, Data expressed as mean ± SEM, n=12-13.
4.3.5 Chronic amitriptyline treatment attenuates SNL-induced mechanical allodynia in OB, but not sham rats

Repeated measures ANOVA revealed an effect of time ($F_{(4,160)} = 19.51$, $P<0.001$) and a time × AMI interaction ($F_{(4,160)} = 3.263, P=0.013$) on 50% mechanical withdrawal thresholds of the ipsilateral hind-paw following SNL (Fig. 4.5A). When compared to pre-SNL values, post-hoc analysis revealed that SNL resulted in reduced mechanical thresholds in both sham and OB animals at all time points examined - indicating SNL-induced mechanical allodynia (Fig. 4.5A). AMI did not alter mechanical thresholds in sham-SNL animals, but OB-SNL AMI-treated animals exhibited increased thresholds when compared to vehicle-treated controls on day 7 only (Fig. 4.5A). Analysis of the area under the curve revealed an effect of OB ($F_{(1,39)} = 6.70, P=0.013$) and AMI ($F_{(1,39)} = 5.26, P=0.027$). OB-SNL AMI-treated animals exhibited increased thresholds when compared to both OB-SNL vehicle-treated and sham-SNL AMI-treated counterparts, indicating an antinociceptive effect (Fig. 4.5B).

As OB animals exhibited slight mechanical allodynia prior to SNL, mechanical thresholds were expressed as a percentage of pre-SNL values, to eliminate this possible confounding effect. Two-way repeated measures ANOVA revealed a significant effect of AMI ($F_{(1,40)} = 6.51, P=0.015$) and time ($F_{(3,160)} = 10.91, P<0.001$) on normalised mechanical withdrawal thresholds of the ipsilateral hind-paw (Fig. 4.5C). Post-hoc analysis revealed that SNL resulted in reduced mechanical thresholds in both sham and OB animals at all time-points examined - indicating SNL-induced mechanical allodynia (Fig. 4.5C). Both AMI-treated sham and OB animals exhibited increased thresholds on day 7 only, when compared to their vehicle-treated counterparts. Analysis of the area under the curve revealed an effect of OB ($F_{(1,40)} = 4.95, P=0.032$) and AMI ($F_{(1,40)} = 9.54, P=0.004$). There was no significant difference between sham-SNL AMI and vehicle-treated controls. In contrast, OB-SNL AMI-treated animals demonstrated increased thresholds when compared to OB-SNL vehicle controls (Fig. 4.5D). There was no effect of OB or AMI treatment on thresholds of the contralateral hind-paw following SNL (see appendix Fig. B.4)
Fig. 4.5 Nociceptive responding to mechanical stimulation of the ipsilateral hind-paw following SNL. (A) 50% mechanical withdrawal thresholds of the ipsilateral hind-paw from pre-SNL to Day 14 post surgery. (B) Area under the curve of 50% mechanical withdrawal thresholds. (C) Mechanical withdrawal thresholds expressed as a percentage of pre-SNL values (D) Area under the curve for normalised withdrawal thresholds. *P<0.05 vs. Sham-SNL-Veh, ++P<0.01 vs. Sham-SNL-AMI, #P<0.05, ##P<0.01 vs. OB-SNL-Veh. Data expressed as mean ± SEM, n=12-13.
4.3.6 Chronic AMI treatment prevents SNL-induced cold allodynia and thermal hyperalgesia in sham, but not OB animals

Repeated measures two-way ANOVA revealed an effect of AMI ($F_{(1,39)} = 5.88$, $P=0.02$) and time ($F_{(1,39)} = 12.64$, $P=0.001$) on latency to respond to application of acetone to the ipsilateral hind-paw. Post-hoc analysis revealed that following SNL, paw withdrawal latency was reduced in sham and OB vehicle-treated animals when compared to pre-SNL levels, indicating SNL-induced cold allodynia (Fig 4.6A). Graphical representation of paw withdrawal latency to acetone post-SNL is also presented in Fig. 4.6B, demonstrating that AMI prevented the SNL-induced cold allodynia in sham-SNL, but not OB-SNL animals.

Friedman’s test revealed an overall effect of group for frequency of paw withdrawals following acetone application to the ipsilateral hind-paw ($Z = -3.32$, $P=0.001$). Post-hoc tests revealed that SNL resulted in increased number of withdrawals in sham and OB vehicle-treated animals when compared to pre-SNL levels, indicating SNL-induced cold allodynia (Fig. 4.6C). Graphical representation of withdrawal frequency to acetone post-SNL is also presented in Fig. 4.6D, demonstrating that AMI prevented the SNL-induced cold allodynia in sham-SNL, but not OB-SNL animals, however a trend in the same direction was noted in OB-SNL AMI-treated animals.

Repeated measures two-way ANOVA revealed an effect of time ($F_{(1,39)} = 173.5$, $P<0.001$) and a time × AMI interaction ($F_{(1,39)} = 5.71$, $P=0.022$) on latency to respond to a noxious heat stimulus prior to and following SNL. Post-hoc analysis revealed that following SNL, paw withdrawal thresholds were reduced in all animals when compared to pre-SNL levels, suggesting thermal hyperalgesia (Fig 4.6C). Sham-SNL AMI-treated animals exhibited increased latency to respond to the heat stimulus when compared to vehicle-treated counterparts, an effect not observed in OB-SNL animals (Fig 4.6D), indicating an anti-nociceptive effect of AMI.
These data indicate that chronic AMI treatment is effective in preventing SNL-induced cold allodynia and thermal hyperalgesia in sham, but not OB animals.

Chronic AMI treatment increased paw withdrawal latency of the contralateral hind-paw in the acetone drop test in sham, but not OB animals, following SNL, indicating an anti-nociceptive effect (Appendix B.5).
Fig. 4.6 Nociceptive responding to thermal (heat and cold) stimulation of the ipsilateral hind-paw following SNL. (A) Paw withdrawal latency to acetone application prior to and after SNL (B) Paw withdrawal latency to acetone post-SNL. (C) Withdrawal frequency to acetone application prior to and after SNL (D) Withdrawal frequency to acetone post-SNL. (E) Paw withdrawal latency to heat prior to and after SNL (F) Paw withdrawal latency to a noxious heat stimulus post-SNL. δδP<0.01, *P<0.01 vs. Pre-SNL values, **P<0.01 vs. Sham-SNL-Veh. Data expressed as mean ± SEM, n=12-13.
4.3.7 OB-SNL animals demonstrate reduced time in the light chamber of the place escape/avoidance paradigm, an effect not altered by amitriptyline

Repeated measures two-way ANOVA revealed an effect of OB ($F_{(1,40)} = 47.810, \ P<0.001$), time ($F_{(5,200)} = 17.483, \ P<0.001$) and a time $\times$ OB interaction ($F_{(5,200)} = 2.571, \ P=0.028$) on time spent in the light-side of the PEAP arena over the 30 minutes of the test, on day 18 following SNL. Post-hoc analysis revealed that OB-SNL vehicle-treated rats spent less time in the light-side of the arena compared to sham-SNL counterparts at all time-points (Fig. 4.7A), an effect not altered by AMI treatment. Although AMI-treated sham-SNL animals spent less time in the light from the 10-15 and 25-30th minutes when compared to vehicle-treated counterparts, this effect failed to reach significance in the ANOVA.

Analysis of total time spent in the light-side of the arena over the 30 minutes revealed an effect of OB (Two way ANOVA: $F_{(1,40)} = 47.81, \ P<0.001$). OB-SNL rats spent less time in the light chamber, compared to their sham counterparts, an effect not altered by AMI (Fig 4.7B).
Fig. 4.7 Affective pain behaviour in the place escape/avoidance paradigm following SNL. (A) Time spent in the light chamber over 5 minute time bins. (B) Total time spend in the light chamber of the PEAP. **P<0.01 vs. Sham-SNL-Veh, +P<0.05, ++P<0.01 vs. Sham-SNL-AMI. Data expressed as mean ± SEM, n=12-13.
4.3.8 Effect of OB and/or chronic AMI administration on the expression of neuroinflammatory markers in the PFC following SNL

There was no effect of side on the expression of neuroinflammatory markers in the PFC or amygdala, thus data for right and left were pooled for subsequent analysis. Data were expressed as a percentage of sham-SNL vehicle-treated controls.

Two-way ANOVA revealed an effect of OB on CD11b ($F_{(1,26)} = 15.95$, $P<0.001$), CD40 ($F_{(1,26)} = 15.84$, $P<0.001$), GFAP ($F_{(1,26)} = 8.19$, $P=0.008$), IL-10 ($F_{(1,24)} = 4.724$, $P=0.04$) and TNFα ($F_{(1,25)} = 5.238$, $P=0.031$) and an effect of AMI treatment on IL-1β ($F_{(1,23)} = 4.942$, $P=0.036$) mRNA expression in the PFC measured on day 20 following SNL (Fig. 4.8).

OB-SNL vehicle-treated animals exhibited increased mRNA expression of markers of microglial (CD11b, CD40) and astrocyte (GFAP) activation, and increased IL-10 mRNA expression compared to sham-SNL counterparts (Fig. 4.8 A-D). The increase in expression of CD11b and CD40 in OB-SNL animals was also present in those that received chronic AMI administration. In comparison, OB-AMI treated animals failed to exhibit the increase in GFAP and IL-10 expression in the PFC observed in vehicle-treated counterparts. AMI did not significantly alter the expression of any of the mediators measured in sham-SNL animals. However, OB-SNL AMI-treated animals exhibited increased TNFα mRNA when compared to sham-SNL AMI-treated counterparts (Fig. 4.8F).

Correlation analysis revealed a significant positive correlation between TNFα expression in the PFC and normalised mechanical withdrawal thresholds (AUC) in the ipsilateral hind-paw ($r=0.3968$, $P=0.04$). There were no other significant correlations between behaviour and inflammatory marker expression.
Fig. 4.8 Expression of inflammatory mediators in the PFC. (A) CD11b (B) CD40 (C) GFAP (D) IL-10 (E) IL-1β (F) TNFα *P<0.05, **P<0.01 vs. sham-SNL-Veh, +P<0.05, ++P<0.01 vs. sham-SNL-AMI. α P<0.05 effect of AMI (ANOVA). Data expressed as mean ± SEM, n=12-13.
4.3.9 Effect of OB and/or chronic AMI administration on the expression of neuroinflammatory markers in the amygdala following SNL

Two-way ANOVA revealed an effect of OB on CD11b ($F_{(1,26)} = 16.98$, $P<0.001$), GFAP ($F_{(1,27)} = 26.54$, $P<0.001$) and IL-10 ($F_{(1,26)} = 4.73$, $P=0.039$) expression in the amygdala (Fig. 4.9 A, C and D). There was no effect of AMI on the expression of neuroinflammatory mediators in the amygdala. Post-hoc analysis revealed that OB-SNL animals exhibited increased CD11b and GFAP expression in the amygdala, an effect not altered by AMI treatment. Although OB overall significantly increased IL-10 expression in the amygdala, further post-hoc analysis indicated that expression of this cytokine in the amygdala of OB-SNL veh/AMI treated animals did not differ from their respective sham counterparts.
Fig. 4.9 (A) CD11b (B) CD40 (C) GFAP (D) IL-10 (E) IL-1β (F) TNFα (G) IL-6 mRNA expression in the amygdala of sham and OB animals following SNL. **P<0.01 vs. sham-SNL-veh animals. *P<0.05, **P<0.01 vs. sham-SNL-AMI, αP<0.05 effect of OB (ANOVA). Data expressed as Mean ± SEM, n=12-13.
4.3.10 Effect of OB and/or chronic AMI administration on monoamine levels in the PFC following SNL

Monoamine concentrations were determined in the PFC by HPLC-ED measured on day 20 post-SNL. Two-way ANOVA revealed an effect of OB on dopamine levels in the PFC ($F_{(1,30)} = 5.06$, $P=0.032$ Fig. 4.10C). *Post-hoc* analysis revealed that OB-SNL vehicle-treated animals exhibited less dopamine in the PFC when compared to sham-SNL counterparts. Dopamine levels in the PFC did not differ between OB-SNL veh/AMI treated animals. There was no significant effect of AMI on the levels on monoamines in the PFC, although an OB × AMI interaction on 5-HT just failed to reach significance ($F_{(1,24)} = 3.47$, $P=0.071$).
Fig. 4.10 Levels of (A) Noradrenaline (B) Dopamine (C) DOPAC (D) 5-HT (E) 5HIAA concentrations in the PFC of sham and OB veh/AMI treated animals following SNL. **P<0.01 vs. Sham-SNL veh. Data expressed as mean ± SEM n = 12-13
4.3.11 Effect of OB and/or chronic AMI administration on monoamine levels in the amygdala following SNL

There was no effect of OB or AMI alone on monoamine levels in the amygdala following SNL. However, two-way ANOVA revealed an OB × AMI interaction on noradrenaline ($F_{(1,33)} = 5.92, P=0.021$) and dopamine ($F_{(1,25)} = 7.2, P=0.013$) levels. OB-SNL vehicle-treated animals demonstrated increased noradrenaline levels in the amygdala when compared to sham-SNL counterparts. Noradrenaline levels in the amygdala were not significantly different between OB-SNL vch/AMI treated animals. (Fig 4.11A). OB-SNL vehicle-treated animals exhibited a trend towards increased dopamine levels, an effect attenuated in AMI-treated OB-SNL animals (Fig 4.11B).

LDOPA and HVA were below the limit of detection for both regions.
Fig. 4.11 Levels of monoamines in the amygdala (A) Noradrenaline (B) Dopamine (C) DOPAC (D) 5HT (E) 5HIAA concentrations in the amygdala **P<0.01 vs. Sham-SNL-Veh, *P<0.05 vs. OB-SNL-Veh. Data expressed as mean ± SEM n = 12-13
4.4 Discussion

The present data demonstrate that chronic amitriptyline treatment attenuated OB-induced hyperactivity in the open field, indicative of an antidepressant-like effect, while not altering nociceptive responding to mechanical or thermal stimuli in either sham or OB rats. SNL induced characteristic mechanical and cold allodynia, and thermal hyperalgesia of the ipsilateral hind-paw of both sham and OB animals. Chronic AMI significantly attenuated SNL-induced mechanical allodynia in OB animals with minimal effect in sham animals. In comparison, chronic AMI attenuated SNL-induced cold allodynia and thermal hyperalgesia in sham, but not OB animals. Thus, amitriptyline differentially modulated nociceptive responding following peripheral nerve injury depending on stimulus modality and the presence or absence of a depressive-like phenotype. OB-SNL animals exhibited enhanced microglial activation (indicated by increased expression of CD11b) in the PFC and amygdala, an effect unaltered by chronic AMI administration. This increase in glial activation is associated with enhanced expression of the anti-inflammatory cytokine IL-10. Chronic AMI did not alter the expression of IL-10 in the amygdala of OB-SNL animals but attenuated its expression in the PFC. Finally, OB-SNL animals exhibited decreased dopamine in the prefrontal cortex and increased noradrenaline in the amygdala, effects not significantly altered by chronic AMI treatment.

4.4.1 Depressive and anxiety-like behaviour are differentially altered by chronic amitriptyline treatment

Correlating with previous data from Chapter 3 and (Roche et al., 2007, Burke et al., 2010), OB animals exhibited a characteristic hyperactivity response in the open field, which was reversed by chronic amitriptyline treatment, confirming the antidepressant-like effect of this TCA in the OB model (Mar et al., 2000, Lumia et al., 1992, van Riezen et al., 1977). Although depressive-like behaviours following SNL were not measured in this study, it has previously been shown that AMI reverses peripheral nerve injury-induced depressive behaviours (Hu et al., 2010, Jesse et al., 2010). In accordance with our previous observations (Chapter 3), OB-SNL rats spent
less time in the light chamber of the PEAP compared with sham controls, an effect not altered by chronic amitriptyline treatment. AMI did not significantly alter time in the light in sham-SNL animals, although a slight trend towards a reduction was noted at certain time-points. As discussed in Chapter 3, this test may be interpreted as a test of anxiety-like behaviour, similar to the light/dark box, containing components of exploration, conflict, and aversive stimuli. Therefore, although AMI is successful at reducing OB-induced depressive-like behaviour and SNL-induced mechanical allodynia, it does not appear to alleviate OB-associated anxiety-related behaviour. This is corroborated by a tendency of OB AMI-treated animals to spend less time in the inner zone of the open field compared to sham counterparts (day 14 and 21 post-OB).

4.4.2 Chronic amitriptyline treatment does not significantly alter OB-associated mechanical allodynia

In evaluating nociceptive responding, our data demonstrate that OB animals exhibit mechanical allodynia, although to a lesser degree than our previous findings (Chapter 3 and Burke et al, 2010). Although we have previously reported that OB animals display cold allodynia (Chapter 3), thermal thresholds to both heat and cold were unchanged following bulbectomy in the current study. Repeated handling combined with the stress of daily i.p. injections of saline may account for the lack of robust alterations observed in the current study. These data are supported by the reduced weight gain over 2 weeks post-surgery in the current study in vehicle-treated animals compared to our other studies (Sham 85±9g vs. 125±5g, OB 75±4g vs. 105±3g). Thermal or mechanical nociceptive responding in either sham or OB animals was not altered by chronic AMI administration. It should be noted that OB-induced mechanical allodynia was not observed in AMI-treated animals (sham-AMI vs. OB-AMI), however the lowest filament response did not differ between vehicle and AMI treated OB animals (OB-veh vs. OB-AMI), indicating no effect of AMI on OB-induced mechanical allodynia. A previous study reported that chronic AMI (21 days, 10mg/kg i.p.) increased swimming behaviour in the rat FST indicating an antidepressant-like effect but failed to alter nociceptive thresholds to
thermal or pressure stimuli applied to the tail or electrical foot-shock (Korzeniewska-Rybicka and Plaznik, 1998). These data indicate that chronic AMI does not alter nociceptive thresholds to mechanical or thermal stimuli in the absence of peripheral nerve injury.

4.4.3 Differential effects of chronic amitriptyline treatment on SNL-induced neuropathic pain-related behaviour in the presence or absence of a depressive-like phenotype

As previously reported (Chapter 3 and Kim and Chung, 1992, Moriarty et al., 2012), SNL resulted in mechanical and cold allodynia, and thermal hyperalgesia in sham and OB animals. There was no difference in responding to mechanical, cold or thermal stimuli in OB vs. sham following SNL. Specifically, the enhancement in cold allodynia observed in OB-SNL animals (Chapter 3) was not observed in the current study. Again, the repeated daily injections may have caused stress on the animals which in turn may have induced alterations in nociceptive processes and masked this effect. Indeed, daily injections of saline have been shown to reduce autotomy behaviour following peripheral nerve deafferentation to a similar degree as AMI, but for a shorter time (Seltzer et al., 1989). Chronic AMI had little effect on SNL-induced mechanical allodynia but rather prevented thermal (cold and heat) hypersensitivity in sham animals, in accordance with previous findings (Esser et al., 2001, Pradhan et al., 2010), indicating modality-specific antinociceptive effects of this treatment in neuropathic pain models. In direct contrast to effects observed in sham animals, AMI did not alter SNL-induced heat hyperalgesia or cold allodynia, but rather attenuated SNL-induced mechanical allodynia in OB animals. Thus, AMI induces differential effects on nociceptive responding following nerve injury depending on the presence or absence of a depressive-like phenotype. Chronic AMI treatment has been shown to attenuate enhanced nociceptive behaviours to electrical stimulation in OB animals (Liang et al., 2011). In addition, subchronic treatment with clomipramine, a tricyclic antidepressant, normalised mechanical but not heat hypoalgesia in learned helpless rats (Li et al., 2013). This study corroborates our findings of differential effect of tricyclic antidepressants on nociceptive responding in a
model of depression, depending on stimulus modality. It is plausible that, in the presence of a depressive-like phenotype, deficits in neurobiological pathways may impede the antinociceptive effect of antidepressant drugs for certain modalities, while facilitating its effect for others. It is well known that thermal and mechanical nociceptive responding are mediated by separate mechanisms (Pradhan et al., 2010, Treede et al., 1992). For instance, thermal hyperalgesia, but not mechanical allodynia following SNL is mediated by C-fibres (Ossipov et al., 1999), while tactile allodynia arises from Aβ- and Aδ-fibres. Spinalization in SNL rats facilitates heat hyperalgesia but suppresses responses to mechanical stimuli, indicating that supraspinal structures have predominantly a tonic inhibitory effect on thermal-evoked spinal reflexes, while supraspinal structures seem to have a predominantly facilitatory effect on responses to mechanical stimuli (Kauppila et al., 1998). Thus, amitriptyline may be more effective in alleviating thermal manifestations of nerve injury in sham animals, perhaps working at a peripheral level, with a supraspinal site of action in OB animals thus attenuating mechanical allodynia. As OB rats have dysfunction of the monoaminergic systems in supraspinal regions, AMI in these animals may result in differential effects than in shams and so modulates neuropathic pain-related behaviour differently, impacting on descending control mechanisms in divergent manners.

4.4.4 The effect of OB and chronic amitriptyline treatment on the expression of neuroinflammatory markers in the prefrontal cortex and amygdala

In accordance with our findings from Chapter 3, OB-SNL vehicle-treated animals exhibited increased expression of microglia and astrocyte markers and the anti-inflammatory cytokine IL-10 in the PFC, however we failed to observe a significant increase in TNFα and IL-1β expression in OB animals in the current study. While microglial activation (indicated by increased expression of CD11b) was unaltered by chronic AMI treatment, increased astrocyte activation and IL-10 expression in the PFC of OB-SNL animals were not seen in animals treated with AMI. Thus, chronic AMI appears to inhibit astrocyte activation and IL-10 mRNA expression in the PFC of OB,
but not sham, animals following SNL. IL-10 is highly expressed in the brain and has been shown to modulate behaviour. The IL-10 receptor is located on microglia, astrocytes and oligodendrocytes (Ledeboer et al., 2002), in the cortex, cerebellum, hippocampus, hypothalamus, and pituitary (Ward et al., 2001). IL-10 has been shown to autoregulate its own expression (Ledeboer et al., 2002), reduce astrogliosis in the cortex (Woiciechowsky et al., 2004, Balasingam and Yong, 1996), and promote survival of glia (Strzele et al., 2002) and neurons (Bachis et al., 2001, Sharma et al., 2011). IL-10 has been implicated in depressive-like behaviours (Mesquita et al., 2008, Bluthe et al., 1999) and thermal nociception (Tu et al., 2003). Of interest, systemic IL-10 has been shown to increase locomotor activity and induce thigmotactic-like behaviour in balb/c mice (Harvey et al., 2006), and as such may underlie the locomotor hyperactivity and anxiety-like behaviour observed in OB animals. In addition, OB-SNL animals exhibited increased TNFα expression following AMI treatment. Thus, the antidepressant-like effect of AMI in OB animals may be related to alterations in neuroinflammatory processes (reduced astrocyte activation and IL-10 and increased TNFα) in the PFC, although further studies are required to evaluate this in detail. TNFα has been shown to inhibit noradrenaline release and is associated with thermal hyperalgesia in the chronic constriction injury (CCI) model of neuropathic pain (Ignatowski et al., 2005, Reynolds et al., 2004). However, TNFα facilitates noradrenaline release in the hippocampus of CCI rats administered amitriptyline (Ignatowski et al., 2005), highlighting dual roles of cytokines in the brain. At later phases following neuronal insult, TNFα expression may be required for repair and recovery in the brain. Indeed, previous studies have demonstrated that TNFα is required for the recovery of neuropathic pain (Nadeau et al., 2011, Ignatowski et al., 2005). Thus, although pro-inflammatory cytokines modulate pain after neuronal injury, they also influence recovery from pain, an effect which may depend on the timing and site of action (Ignatowski et al., 2005, Sadeghi et al., 2011). A mechanism of action of antidepressant drugs may be via the restoration of the cytokine balance. In fact, AMI has been shown to increase total TNFα levels in brain tissue homogenates.
Chapter 4 – Effect of amitriptyline in OB-SNL

(Reynolds et al., 2004). In the current study, there was a tendency for AMI to increase noradrenaline levels in the PFC of OB-SNL animals. In addition, TNFα expression was correlated with normalised mechanical withdrawal thresholds of the ipsilateral hind-paw, suggesting that the enhancement of TNFα by AMI may be a potential mechanism for the alleviation of mechanical allodynia in OB animals.

In Chapter 3, we observed increased expression of microglial and astrocyte markers in the amygdala of OB non-SNL animals, but not following SNL. In comparison, the current study demonstrated that OB-SNL was associated with an increase in microglial and astrocyte activation and IL-10 expression in the amygdala. While these effects are similar to those observed in the PFC, the discrepancy between this data and that presented in Chapter 3 may be related to the slightly different experimental timeline or effects of repeated injections. While AMI attenuated the increase in GFAP and IL-10 expression in the PFC, this was not observed in the amygdala. Thus, AMI induces differential effects on neuroinflammatory processes in discrete brain regions. Similar to the PFC, there was a strong trend towards increased TNFα expression in OB-SNL animals following AMI treatment.

4.4.5 The effect of OB and chronic amitriptyline treatment on the levels of monoamines in the prefrontal cortex and amygdala

The antidepressant activity of TCAs has been attributed to their ability to enhance monoamine neurotransmission and by the same mechanism this may be how they elicit their analgesic effects. Overall, there were minimal effects of chronic AMI administration on monoamine levels in the two brain regions examined herein, although AMI resulted in a strong trend towards increased noradrenaline and dopamine in the amygdala of sham, but not OB animals following SNL. The lack of effect of AMI on monoamine levels may not be surprising due to alterations in the expression of autoreceptors which occurs as a result of sustained elevations of neurotransmitters and changes in synaptic efficiency. For example, chronic AMI treatment reduces α2 adrenergic receptor sensitivity (Charney et al., 1983) and increases serotonin receptor sensitivity (Friedman et al., 1983), restoring deficits in
monoaminergic neurotransmission, which may have implications for the descending inhibition/facilitation of pain. Thus, the effect of chronic AMI treatment is most likely due to neuronal changes induced by high levels of monoamines, which accounts for their delayed onset of action as seen in depression. These data again indicate that TCAs may modulate monoamine levels differently in intact and OB animals, which may account for the differential effects of amitriptyline on nociceptive responding in the presence of bullectomy. Nonetheless, the data presented in the current chapter show that modulation of the monoaminergic system using amitriptyline attenuates depressive-like behaviour and SNL-induced mechanical allodynia in the OB rat.

4.5 Conclusion

The data presented in the current chapter indicate that chronic AMI treatment prevents the development of depressive-like behaviour in the OB rat, and alters nociceptive behaviour following peripheral nerve injury differentially in the presence or absence of a depressive-like phenotype. In addition, chronic antidepressant treatment differentially modulates inflammatory mediator gene expression in sham and OB animals following SNL. Taken together, these data support a role for the monoaminergic system in behavioural modulation of affect, pain and the interaction between these conditions.
4.6 Summary of aims and subsequent findings

(1) Examined if chronic AMI treatment alters depressive-like behaviour in OB
   (i) Yes, chronic AMI prevented depressive-like behaviour in the OB rat

(2) Determined if chronic AMI alters nociceptive responding to mechanical and thermal stimuli, and if such an effect is altered in OB animals
   (ii) No, chronic AMI treatment did not alter nociceptive responding in sham or OB

(3) Determined the effect of chronic AMI on SNL-induced mechanical and thermal allodynia (sham animals)
   (iii) Chronic AMI prevented SNL-induced thermal and cold, but not mechanical, hypersensitivity in sham animals.

(4) Investigated if chronic AMI induces differential effects on SNL-induced allodynia in sham and OB rats
   (iv) Yes, chronic AMI prevented SNL-induced mechanical, but not cold/heat, hypersensitivity in OB rats.

(5) Determined if chronic AMI treatment alters affective pain behaviour following SNL, and if this differed in OB animals
   (v) No, chronic AMI did not alter affective pain behaviour in sham or OB

(6) Evaluated the effect of chronic AMI on levels of monoamines and inflammatory mediators in discrete brain regions associated with affective and nociceptive processing (PFC and amygdala)
   (vi) Chronic AMI treatment differentially modulated inflammatory mediator gene expression and monoamine levels in the PFC/amygdala of sham and OB animals following SNL.
Chapter 5

The effect of microglial inhibition on depressive-like behaviour and nociceptive responding prior to and following peripheral nerve injury in the OB rat: accompanying alterations in neuroinflammatory mediators
5.1 Introduction

Glia, the term derived from the Greek word for glue, are the non-neuronal cells of the central nervous system. Originally believed to play a supportive and protective role to neurons, it is now established that glia are active participants involved in neurotransmission, synaptic plasticity, and development of the healthy brain. A wealth of evidence implicates glial cell activation and inflammatory processes in the pathogenesis of depression and chronic pain (for review see Chapter 1 and Gosselin et al., 2010b, Maes et al., 2011), and therefore it is not surprising that such processes may be involved in the interaction between these disorders.

Minocycline is a second-generation tetracycline derivative that inhibits microglial activity independent of its antibiotic effects (Tikka et al., 2001, Tikka and Koistinaho, 2001) and thus is a useful tool to investigate the mechanisms underlying diseases accompanied by microglial activation. It is a small lipophilic molecule which readily crosses the blood-brain barrier (Colovic and Caccia, 2003), and is well-tolerated clinically with a low side effect profile (Seukeran et al., 1997), properties that make it an excellent candidate for the treatment of CNS disorders with an inflammatory component (Yong et al., 2004). Administration of this agent inhibits inflammatory responses by immune cells (T-cells, macrophages and microglia) by reducing \textit{in vitro} pro-inflammatory cytokine production/release, nitric oxide release, iNOS gene expression (Giuliani et al., 2005, Suk, 2004, Amin et al., 1996) and by preventing glutamate-induced excitotoxicity (Tikka and Koistinaho, 2001, Pi et al., 2004). Minocycline has been shown to induce neuroprotective effects \textit{in vivo} in animal models of spinal cord injury (Stirling et al., 2004, Teng et al., 2004), cerebral ischemia (Yrjanheikki et al., 1999, Arvin et al., 2002, Morimoto et al., 2005, Wang et al., 2003), Parkinson's disease (Du et al., 2001, Wu et al., 2002), Huntington's disease (Chen et al., 2000) and amyotrophic lateral sclerosis (Zhu et al., 2002). Some of its neuroprotective actions have been attributed to preventing cytochrome c leakage from mitochondria (Zhu et al., 2002; Teng et al., 2004), and inhibition of caspases (Chen et al., 2000)
and matrix metalloproteases (Greenwald et al., 1992), effects which may result from interference with upstream processes involved in microglial activation. Indeed, an abundance of evidence supports that the neuroprotective effects of minocycline are mediated via inhibition of microglial activation (Yrjanheikki et al., 1998, Tikka et al., 2001, Tikka and Koistinaho, 2001, Bye et al., 2007, Cho et al., 2011, Cho et al., 2006, Guasti et al., 2009, Krady et al., 2005, Hinwood et al., 2012).

There is some clinical evidence that minocycline may have an antidepressant effect. Depressed patients who had been on a stable treatment of selective serotonin reuptake inhibitors achieved greater than a 50% reduction in depressive symptoms after supplementing treatment with minocycline for 6 weeks (Miyaoka et al., 2012). In addition, minocycline has been reported to have antipsychotic properties and may be beneficial for the negative symptoms of schizophrenia (Miyaoka et al., 2007, Chaudhry et al., 2012). A clinical trial is currently underway examining the efficacy of minocycline in combination with aspirin for the treatment of bipolar disorder (Savitz et al., 2012). A single but highly interesting case-study reported that a patient with bipolar depression who only partially responded to 4 weeks of clomipramine treatment exhibited a significant reduction in depressive symptoms and achieved resolution of facial pain after 3-4 days of adjunct minocycline treatment (Levine et al., 1996). Although further studies are required, this may indicate that such a treatment strategy could alleviate both primary depression and associated comorbid pain. These clinical data have been supported by a number of rodent studies demonstrating antidepressant-like effects of minocycline (Table 5.1), however the effect of minocycline on nociceptive responding in models of depression has not been examined. Systemic minocycline treatment has been shown to decrease immobility in the forced swim test in one report (Molina-Hernandez et al., 2008a) but not another study (Deak et al., 2005), which may be due to the difference in timing and dose of administration (see Table 5.1). However, when the immune system is stimulated, minocycline may have a greater potential to exert an antidepressant effect by reducing inflammatory signalling. In fact, LPS-induced anhedonia and
social behavioural deficits (Henry et al., 2008a) and LPS-induced increases in immobility in the forced swim and tail suspension tests (O'Connor et al., 2009b) are prevented by pretreatment with minocycline. Systemic minocycline has been shown to decrease locomotor activity (Molina-Hernandez et al., 2008a) but also prevent LPS-induced reductions in locomotor activity (O'Connor et al., 2009b). Chronic minocycline treatment has been shown to prevent OB-induced hyperactivity and spatial memory deficits (Borre et al., 2012b), and attenuate chronic stress-induced deficits in spatial working memory (Hinwood et al., 2012). Central administration (i.c.v.) of minocycline has been shown to improve conditioned avoidance in the learned helplessness paradigm (Arakawa et al., 2012). Its effects have been associated with modulation of neurotransmitter pathways and cytokines in the brain. For example, minocycline reduces LPS-induced proinflammatory cytokine expression and IDO in the cortex and hippocampus (Henry et al., 2008a), limits exaggerated neuroinflammation in aged mice (Henry et al., 2008a), normalises the kynurenine/tryptophan ratio (O'Connor et al., 2009b), prevents foot-shock stress-induced increases in hypothalamic IL-1β (Blandino et al., 2006), and increases dopamine levels in the amygdala of learned helplessness animals (Arakawa et al., 2012). A subthreshold dose of minocycline synergised with subthreshold doses of desipramine and putative antidepressants such as glutamate receptor antagonists to elicit an antidepressant-like effect in the forced swim test (Molina-Hernandez et al., 2008a, Molina-Hernandez et al., 2008b), which may suggest that minocycline modulates monoaminergic or glutamatergic neurotransmission.

Several studies have examined the role of microglia in the development and maintenance of persistent pain states, utilising minocycline as a tool in this regard. Clinically, there is evidence to suggest that minocycline may be beneficial in reducing disease activity in rheumatoid arthritis (O'Dell et al., 2001, Kloppenburg et al., 1994, Langevitz et al., 2000). In addition, minocycline has an anti-hyperalgesic effect when given acutely to sciatica patients (but has no effect on the response to intradermal capsaicin in these patients) (Sumracki et al., 2012), and topical minocycline has been shown to
reduce pain after local administration following dental surgery (Gelesko et al., 2011). A recent study reported that perioperative minocycline administration for 8 days does not improve persistent pain after lumbar discectomy, but the authors reported that minocycline was efficacious in a subgroup of patients with deep spontaneous pain prior to surgery (Martinez et al., 2013). Minocycline has been extensively examined in animal models of nociception and pain (Table 5.1). Treatment with minocycline does not appear to alter nociceptive thresholds to thermal stimuli (Bastos et al., 2007, Mika et al., 2009, Padi and Kulkarni, 2008). Acetic acid-induced visceral hyperalgesia was reported to be attenuated by minocycline in one study (Cho et al., 2012), but not another (Padi and Kulkarni, 2008), an effect which may be due to the timing of administration (60 min vs. 30 min) as minocycline levels have been shown to peak in the plasma of rats 1-2 hours post-treatment (Colovic and Caccia, 2003). Minocycline inhibits inflammatory nociception in preclinical models such as the formalin test (Leite et al., 2011, Bastos et al., 2007, Hua et al., 2005, Pabreja et al., 2011, Li et al., 2010) and carrageenan-induced hyperalgesia (Hua et al., 2005). Pretreatment of rodents with minocycline has consistently been shown to prevent the development of neuropathic pain-related behaviour in models including SNL, partial sciatic nerve ligation, and spinal nerve transection (Mei et al., 2011, Nie et al., 2010, Raghavendra et al., 2003, Lin et al., 2007), an effect which appears to be due to suppression of microglial activation and proinflammatory cytokines release in the spinal cord (Raghavendra et al., 2003, Zanjani et al., 2006, Piao et al., 2006, Pabreja et al., 2011, Guasti et al., 2009). However, it should be noted that minocycline treatment fails to reverse established allodynia/hyperalgesia, suggesting a role for microglia in the initiation and development of neuropathic pain behaviour, but not the maintenance of this pain state (Raghavendra et al., 2003). As microglial activation and enhanced inflammatory cytokines have been observed in anatomical locations distant from the site of lesion, such as the rostral ventromedial medulla, locus coeruleus, hypothalamus, periaqueductal grey, PFC and hippocampus (Al-Amin et al., 2011, Ignatowski et al., 1999, Apkarian et al., 2006, Takeda et al., 2009a, Wei et
al., 2008), a mechanism of action of minocycline may occur via reduction in inflammation in brain regions that process pain. Moreover, minocycline microinjection into the RVM inhibited CCI-induced hyperalgesia and allodynia when administered at the early phase (day 3) but not the late phase (day 14) (Wei et al., 2008). Taken together, these data indicate a role for microglial activation at both spinal and supraspinal sites in the development, but not maintenance, of neuropathic pain states. Thus, preventing microglial activation has been shown to attenuate depression and persistent pain. However, the role of microglia in the interaction between depression and chronic pain has been under investigated.
### Chapter 5 – Effect of minocycline in OB-SNL

<table>
<thead>
<tr>
<th>Model</th>
<th>Dose</th>
<th>Duration</th>
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<th>Result</th>
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<tr>
<td><strong>Depression model</strong></td>
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<tr>
<td>FST</td>
<td>50, 60, 80 mg/kg</td>
<td>23, 5 and 1 h prior to tests</td>
<td>i.p.</td>
<td>Mino (60-80 mg/kg, immobility in FST and LMA in the open field test)</td>
<td>(Molina-Hernandez et al., 2008a)</td>
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<tr>
<td>FST</td>
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<td>15 mins prior to testing</td>
<td>Intra-NAcc</td>
<td>Intra-NAcc mino (1.0-2.5 μg) immobility in the FST and LMA in the open field</td>
<td>(Molina-Hernandez et al., 2008b)</td>
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<tr>
<td>FST</td>
<td>20 or 40 mg/kg</td>
<td>1 hour prior to test</td>
<td>i.p.</td>
<td>No antidepressant effect in the FST</td>
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<td>LPS-induced depression</td>
<td>50 mg/kg</td>
<td>3 days prior to LPS</td>
<td>i.p.</td>
<td>Attenuated LPS-induced anhedonia, reductions in social exploration, weight loss and anorexia</td>
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<tr>
<td>LPS-induced depression</td>
<td>50 mg/kg</td>
<td>2 days prior to and on the day of LPS admin</td>
<td>i.p.</td>
<td>Prevented LPS-induced immobility in FST &amp; TST and attenuated reductions in body weight and locomotor activity</td>
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<tr>
<td>Olfactory bulbectomy</td>
<td>50 mg/kg</td>
<td>4 weeks</td>
<td>i.p.</td>
<td>Attenuates OB-induced hyperactivity</td>
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<tr>
<td>Chronic restraint stress</td>
<td>40 mg/kg/day</td>
<td>24 h prior to stress and for the duration of the stress protocol (21 days)</td>
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<td>Prevented deficits in spatial working memory</td>
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<tr>
<td>Learned helplessness</td>
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<td>4 days prior to test</td>
<td>i.c.v.</td>
<td>Prevented impairments in conditioned avoidance</td>
<td>(Arakawa et al., 2012)</td>
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<td><strong>Pain model</strong></td>
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<td>Acute Pain Tests</td>
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<td>Hot plate test</td>
<td>1000 mg/ kg</td>
<td>1 h prior to test</td>
<td>i.p.</td>
<td>No effect on thermal nociceptive behaviour</td>
<td>(Bastos et al., 2007)</td>
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<tr>
<td>Tail flick test</td>
<td>30 mg/kg</td>
<td>Twice daily for 12 days, 60 mins before testing</td>
<td>i.p.</td>
<td>No effect on thermal nociceptive behaviour</td>
<td>(Mika et al., 2009)</td>
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<tr>
<td>Acetic acid, zymosan, tail immersion test</td>
<td>30 or 100 mg/kg</td>
<td>30 mins prior to testing</td>
<td>i.p.</td>
<td>No effect on nociceptive behaviour</td>
<td>(Padi and Kulkarni, 2008)</td>
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<tr>
<td>Acetic acid</td>
<td>4, 10, 40 mg/kg</td>
<td>60 mins prior to testing</td>
<td>i.p.</td>
<td>Attenuated visceral hyperalgesia</td>
<td>(Cho et al., 2012)</td>
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<td><strong>Inflammatory pain models</strong></td>
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<td>LPS-induced hyperalgesia</td>
<td>25 mg/kg</td>
<td>Once daily for 3 days prior to LPS</td>
<td>i.p.</td>
<td>Attenuated LPS-induced thermal and mechanical hyperalgesia</td>
<td>(Yoon et al., 2012)</td>
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<tr>
<td>Carrageenan</td>
<td>20, 60 and 120 μg</td>
<td>15-20 mins prior to testing</td>
<td>t.t.</td>
<td>Attenuated carrageenan-evoked thermal hyperalgesia</td>
<td>(Hua et al., 2005)</td>
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<tr>
<td>Carrageenan</td>
<td>100 mg/kg</td>
<td>1 hour prior to testing</td>
<td>i.p.</td>
<td>Attenuated carrageenan-evoked mechanical allodynia</td>
<td>(Bastos et al., 2007)</td>
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<tr>
<td>Carrageenan</td>
<td>60 mg/kg</td>
<td>15-20 mins prior to testing</td>
<td>i.p.</td>
<td>Delayed onset of thermal hyperalgesia (by 30 mins)</td>
<td>(Hua et al., 2005)</td>
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<tr>
<td>Carrageenan</td>
<td>25 or 50 μg</td>
<td>15 min prior to testing</td>
<td>RVM</td>
<td>Attenuated carrageenan-evoked thermal hyperalgesia</td>
<td>(Roberts et al., 2009)</td>
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<td>Formalin (2.5%)</td>
<td>20, 60 and 120 μg</td>
<td>15-20 mins prior to testing</td>
<td>t.t.</td>
<td>Antinociceptive effect in 2nd phase formalin test.</td>
<td>(Hua et al., 2005)</td>
</tr>
<tr>
<td>Formalin (2.5%)</td>
<td>60 mg/kg</td>
<td>15-20 mins prior to testing</td>
<td>i.p.</td>
<td>Antinociceptive effect in 1st, not 2nd phase formalin test.</td>
<td>(Hua et al., 2005)</td>
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<tr>
<td>Formalin (5%)</td>
<td>15, 30 and 45 mg/kg</td>
<td>1h prior to formalin</td>
<td>i.p.</td>
<td>Antinociceptive effect in 2nd phase of formalin test.</td>
<td>(Cho et al., 2006)</td>
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<td>Formalin (1%)</td>
<td>5, 10, 25 mg/kg</td>
<td>30 mins prior to testing</td>
<td>i.p.</td>
<td>Antinociceptive effect in 2nd phase of formalin test.</td>
<td>(Leite et al., 2011)</td>
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<tr>
<td>Formalin (2.5%)</td>
<td>100 mg/kg</td>
<td>1h prior to testing</td>
<td>i.p.</td>
<td>Antinociceptive effect in 2nd phase of formalin test.</td>
<td>(Bastos et al., 2007)</td>
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| Formalin (5%) (2 trt: day 1 - 5%, day 7 - 1%) | 40 mg/kg      | 7 day trt beginning 1 hour prior to 1st formalin trt | i.p.           | Attenuated enhanced nociceptive behaviour in rats receiving 2 trt formalin regime | (Li et al., 2010)
### Chapter 5 – Effect of minocycline in OB-SNL

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<th>Formalin (2%)</th>
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<td>L5 spinal nerve transection</td>
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<td>Pre-trt: 1h pre-surgery &amp; daily to day 10 Post-trt regime: Beginning on day 5 post-injury</td>
<td>i.p.</td>
<td>Pre-trt mino attenuated L5 induced mechanical hyperalgesia and allodynia Post-trt: No effect</td>
<td>(Raghavendr a et al., 2003)</td>
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<td>L5/L6 SNL</td>
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<td>i.p.</td>
<td>Mino reduced SNL-induced mechanical allodynia at days 5, 10 and 14 (but not day 1-3)</td>
<td>(Guasti et al., 2009)</td>
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<td>L5/L6 SNL</td>
<td>2 and 6 μg/h</td>
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<td>L5 SNL</td>
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<td>t.t.</td>
<td>20 &amp; 40μg mino partially while 60μg fully attenuated SNL-induced mechanical allodynia</td>
<td>(Pu et al., 2012)</td>
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<td>L5 SNL</td>
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<td>4 days</td>
<td>t.t.</td>
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<td>(Mei et al., 2013)</td>
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<td>L5 SNL</td>
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<td>Pre-trt: Before surgery then for 6 days Post-trt: Day 3 post-SNL then for 5 days</td>
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<td>L5 SNL</td>
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<td>t.t.</td>
<td>Mino attenuated SNL-induced mechanical allodynia when administered on day 1, 3, &amp; 7 but not 10 or 21</td>
<td>(Mei et al., 2011)</td>
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<td>Partial sciatic nerve ligation</td>
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<td>Prevented development of mechanical allodynia (measured day 10 post surgery)</td>
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<td>CCI</td>
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<td>Pre-trt regime: 2h pre-surgery and daily for 7 days post-CCI Post-trt regime I: day 4 post-CCI Post-trt regime II: repeated from day 7 post-CCI</td>
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<td>Pre-trt: Mino partially prevented development of CCI-induced cold allodynia and mechanical hyperalgesia Post-trt I: No effect Post-trt II: no effect</td>
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<tr>
<td>CCI</td>
<td>5, 10, 20 and 40 mg/kg</td>
<td>1h pre-surgery and daily until day 14</td>
<td>i.p.</td>
<td>10, 20 and 40 mg/kg mino prevented CCI-induced thermal hyperalgesia, mechanical and cold allodynia</td>
<td>(Zanjani et al., 2006)</td>
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<td>CCI</td>
<td>15, 30, and 50 mg/kg</td>
<td>16h and 1h pre-surgery, then twice daily for 7 days</td>
<td>i.p.</td>
<td>Attenuated CCI-induced mechanical allodynia and cold hyperalgesia</td>
<td>(Mika et al., 2007)</td>
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<tr>
<td>CCI</td>
<td>10, 20 and 40 mg/kg</td>
<td>Post-injury (Day 6-13)</td>
<td>i.p.</td>
<td>40 mg/kg attenuated CCI-induced thermal hyperalgesia and mechanical allodynia on day 13</td>
<td>(Nazemi et al., 2012)</td>
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<tr>
<td>STZ neuropathy</td>
<td>40 and 80 mg/kg</td>
<td>Once daily for 2 weeks (beginning 2 wk after STZ when hyperalgesia were established)</td>
<td>i.p.</td>
<td>7 (but not 3) days of treatment 80 mg/kg prevented the development of cold allodynia and thermal hyperalgesia</td>
<td>(Pabloja et al., 2011)</td>
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</tr>
<tr>
<td>Partial sciatic nerve injury</td>
<td>4 μL</td>
<td>1 hour prior to surgery and every day for 7 days after</td>
<td>t.t.</td>
<td>Mino partially attenuated mechanical allodynia (day 4-8) and hyperalgesia (day 6-8); and thermal hyperalgesia (day 3-7)</td>
<td>(Narita et al., 2006c)</td>
<td></td>
</tr>
<tr>
<td>Facial nerve transection</td>
<td>15 or 30 mg/kg</td>
<td>1 hour prior to surgery and every day for 14 days after</td>
<td>i.p.</td>
<td>Mino dose-dependently attenuated mechanical hypersensitivity, sustained for a further 2 wks after stopping trt</td>
<td>(Piao et al., 2006)</td>
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</tr>
<tr>
<td>Sciatic inflammatory neuropathy</td>
<td>50 or 100 μg zymosan</td>
<td>24h after perisietic</td>
<td>t.t.</td>
<td>Mino (100 μg) attenuated zymosan-induced mechanical allodynia for 24hrs post injection</td>
<td>(Ledeboer et al., 2005)</td>
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</tr>
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Table 5.1 Summary of studies demonstrating antidepressant and antinociceptive effects of minocycline. CCI chronic constriction injury; FST forced swim test; i.t. intrathecal; i.p. intraperitoneal; LMA locomotor activity; LPS lipopolysaccharide; Mino Minocycline; SNL spinal nerve ligation; RVM rostral ventromedial medulla; TST tail suspension test; trt treatment.
Chapter 3 and 4 revealed alterations in the expression of microglial markers and neuroinflammatory mediators in the OB-SNL rat, effects associated with differential behavioural changes between sham and OB rats. Moreover, chronic amitriptyline treatment differentially altered nociceptive behaviour following SNL and the expression of neuroinflammatory mediators in the PFC. Thus, the current study aimed to examine the effect of inhibition of microglial activation on depressive-like behaviour and nociceptive responding prior to and following peripheral nerve injury in the OB rat. A further aim of this study was to examine if chronic minocycline treatment altered mRNA expression of genes encoding inflammatory mediators in the spinal cord, PFC and amygdala.

5.1.1 Hypothesis and aims

Hypothesis
Chronic, but not acute, minocycline would elicit an antidepressant-like effect in OB rats and prevent SNL-induced allodynia, effects accompanied by altered expression of neuroimmune mediators in the spinal cord, PFC and amygdala

Aims - Study 1

1. Determine the effect of chronic inhibition of microglial activation on depressive-like behaviour in the OB rat
2. Examine the effect of chronic minocycline administration on nociceptive responding to mechanical and thermal stimuli
3. Evaluate if OB-related mechanical allodynia is altered by chronic minocycline administration
4. Investigate the effect of chronic minocycline on SNL-induced mechanical and cold allodynia, and examine if chronic minocycline alters SNL-induced allodynia/hyperalgesia differentially in sham vs. OB rats
5. Evaluate the effect of chronic minocycline on the mRNA expression of genes encoding inflammatory mediators in the PFC, amygdala and spinal cord following SNL

Study 2

A further study evaluated if an acute systemic administration of minocycline could induce similar behavioural effects to that observed following chronic treatment. Thus, in order to address if microglial inhibition in an acute time frame could attenuate OB-induced hyperactivity and alter nociceptive responding prior to and following SNL in this model, minocycline was administered once off on day 14 post-OB.

Specifically, these aims were as follows:

1. Determine if acute minocycline treatment alters OB-induced hyperactivity

2. Establish if acute minocycline treatment alters nociceptive responding to mechanical stimuli prior to nerve injury, and if such an effect is altered in OB animals

3. Examine if acute minocycline pretreatment alters the development (day 1) of SNL-induced allodynia differentially in sham vs. OB rats
5.2 Materials and methods

5.2.1 Animals

Male Sprague Dawley rats weighing 180-220g (Study 1) and 200-225g (Study 2) on arrival were obtained from Charles River UK. Animals were singly housed in standard conditions and protocol approved as described in Chapter 2, Section 2.1. Behavioural testing was carried out during the light phase and animals were handled and weighed daily.

5.2.2 Drug administration

Study 1: Chronic minocycline administration

Minocycline Hydrochloride (Hovione Ltd, Loures, Portugal) was dissolved in tap water to a concentration of 1mg/ml (solution pH 5.2). Treatment was administered orally via drinking water which was available ad libitum, and control groups received tap water. Water bottles were weighed daily to determine fluid intake. Fresh drug stock was prepared and replaced every 3-4 days. This protocol was based on evidence for minimal degradation of minocycline in solution at room temp over 7 days (Smith et al., 2003, Pearson and Trissel, 1993).

Study 2: Acute minocycline administration

Minocycline Hydrochloride was made up in sterile saline (0.9% w/v) fresh, and administered systemically by intraperitoneal injection at a dose of 80mg/kg in an injection volume of 2ml/kg, 3 hours prior to the open field. The dose was based on the average intake of minocycline in Study 1 of the current chapter, and the route of administration was based on the previous antidepressant effects at this dose and route (Molina-Hernandez et al., 2008a). The timing was based on the requirement to detect an antidepressant effect in OB animals in the open field without altering locomotor activity in intact (sham) animals (Kofman et al., 1990). Furthermore, this dose is in excess of that reported to inhibit microglial activation when administered prior to peripheral nerve injury (Raghavendra et al., 2003, Guasti et al., 2009).
5.2.3 Experimental design

5.2.3.1 Study 1

The experimental design is presented in Figure 5.1 and was similar to that described in Chapter 4. Essentially, male Sprague Dawley rats were tested in the open field (Section 2.4.1.1) prior to surgery to ensure no between-group differences in locomotor activity upon allocation of animals to one of four groups: Sham-Water (n=11), Sham-Mino (n=13), OB-Water (n=12), OB-Mino (n=12). Minocycline (Mino) or vehicle (water) treatment began 24h prior to sham/OB surgery (Section 2.2), and continued until the end of the experiment. Following sham or OB surgery, animals were allowed two weeks to recover before re-exposure to the open field test (day 14) followed by nociceptive testing (Section 2.4.2) to mechanical (von Frey: day 15), cold (acetone drop test: day 15) and heat (Hargreaves: day 16) stimuli. Seventeen days post-bulbectomy, L5/L6 Spinal nerve ligation (SNL) surgery (Section 2.3) was carried out on all animals, and mechanical withdrawal thresholds were assessed on days 1, 3, 7, 10 and 14 post-SNL. Thermal nociception was assessed in the acetone drop test and Hargreaves test on day 14 and 17 post-SNL, respectively. Finally, animals were re-tested in the open field on day 19 post-SNL. Twenty-four hours following the last behavioural assessment, animals were sacrificed by rapid decapitation. The brains and spinal cords were removed, the PFC and amygdala were dissected out, snap frozen on dry ice and stored at -80°C until mRNA expression of inflammatory markers was determined using qRT-PCR as described in Chapter 2 (Section 2.7). Prior to RNA isolation, spinal cords (kept cold on dry ice) were separated into dorsal and ventral, and the dorsal half was divided into ipsilateral and contralateral for analysis. Minocycline levels in the cortex were determined by liquid chromatography (LC)-mass spectrometry as described in section 5.2.4 of the current chapter.

One OB-Water rat was eliminated from analysis after frontal cortex damage was observed upon gross inspection at the end of the experiment. Three animals (sham-water n=1 and sham-Mino n=2) died in the days following
SNL surgery. Final numbers were therefore; sham-water (n=10), sham-mino (n=12), OB-water (n=11) and OB-mino (n=12).

**Fig. 5.1 Experimental design – Study 1.** OB olfactory bulbectomy; VF von Frey test; HG Hargreaves test; AT acetone drop test; SNL Spinal nerve ligation
5.2.3.2 Study 2

Animals were tested in the open field the day prior to surgery to ensure no differences in baseline locomotor activity (Chapter 2, Section 2.4.1.1). Animals were subsequently divided into one of four groups: Sham-veh (vehicle, n=10), Sham-Mino (minocycline, n=7), OB-Veh (n=11) and OB-Mino (n=9). OB or sham surgery was then carried out as previously described (Section 2.2). One OB animal died from haemorrhage the day after surgery. Two OB-Veh animals were eliminated from analysis due to some bulb remaining which was observed on gross inspection at the end of the study. One OB-Mino rat was removed due to frontal cortex damage. Final numbers were therefore: Sham-Veh (n=10), Sham-Mino (n=7), OB-Veh (n=9) and OB-Mino (n=8). Fourteen days later, minocycline (80 mg/kg) or vehicle (sterile saline) was administered i.p. 3 hours prior to the open field. Locomotor activity (distance moved, cm), time in the inner zone, and rearing and grooming behaviours were examined in the open field using EthoVision® XT software (Appendix C). Directly after the open field, mechanical withdrawal thresholds were examined in the von Frey test (Section 2.4.2.1) to determine the effect of OB and/or acute minocycline on mechanical responding. Directly following the von Frey test, SNL surgery (Section 2.3) was carried out on all animals. One animal was euthanized due to L4 damage (OB-mino n=1). Twenty-four hours following SNL, mechanical withdrawal thresholds were re-examined to determine the effect of an acute dose of minocycline on the development of SNL-induced mechanical allodynia in sham and OB animals. Following von Frey testing, animals were sacrificed by rapid decapitation.
Fig. 5.2 Experimental design Study 2. OB Olfactory bulbectomy; SNL Spinal nerve ligation, i.p. intraperitoneal
5.2.4 Mass spectrometry for determination of minocycline levels in cortical tissue

In order to confirm and determine that minocycline administered in drinking water was capable of crossing the blood brain barrier and inhibiting microglial activation, levels of minocycline were assessed using liquid chromatography mass spectrometry in cortical tissue at the end of the study (day 38 of treatment). Stock aqueous solutions of minocycline (40μg/ml) and its internal standard tetracycline (Sigma, 40μg/ml) were prepared. A standard curve was prepared from stock solutions (minocycline) in 100% acetonitrile, giving a 6-point curve in the range of 3μg to 2.9ng, with a fixed amount of tetracycline internal standard in each standard (4μg). Tissue homogenising buffer was prepared by diluting tetracycline stock solution 1:4 with 100% acetonitrile to give a final concentration of 4μg tetracycline/400μl. Cortical tissue samples were sonicated in 400μl of homogenising buffer. Following sonication, homogenates were centrifuged at 14,000g for 15 minutes at 4°C and the supernatant collected and evaporated to dryness in a centrifugal evaporator. Lyophilised samples were resuspended in 40μl of 5% acetonitrile and 1μl was injected onto a Zorbax® SB C18 column (150 × 0.5 mm internal diameter) from a cooled autosampler maintained at 4°C. Mobile phases consisted of solvent A (0.1% formic acid (v/v) in water) and solvent B (0.1% (v/v) formic acid in acetonitrile) maintained at a flow rate of 12μl per minute. Reversed-phase gradient elution began initially at 2% B and over 10 minutes was ramped linearly up to 100% B and held at 100% B for a further 10 minutes. Under these conditions, minocycline and tetracycline eluted at 11.8 and 12.3 minutes, respectively. Analyte detection was carried out in electrospary-positive ionisation and multiple reaction monitoring (MRM) mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). The transitions monitored were mass/charge (m/z) ratios, parent 458 > daughter 441 and 445 > 410 with a collision energy of 22eV and 18eV for minocycline and tetracycline, respectively. Quantitation of the minocycline analyte was performed by determining the peak height response of minocycline against
its corresponding internal standard. The amount of minocycline in unknown samples was calculated from the minocycline/internal standard peak height response ratio using a 6-point calibration curve, as described above. Linearity was determined over a range of 3μg to 2.9ng. Results were expressed as μg/g tissue.

![Fig 5.3](image)

**Fig 5.3 Output from Agilent Masshunter Quantitative Analysis program.** Chromatogram retention time for (A) minocycline and (B) the internal standard tetracycline. (C) Relative concentration ([minocycline] / [tetracycline]) / relative response (peak height minocycline in sample / peak height of tetracycline in sample).
5.2.5 Statistical analysis

PASW 18 statistical program was used to analyse all data. All data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene’s tests respectively. The statistical significance of differences between groups was assessed using t-tests, two-way analysis of variance (ANOVA), using factors of OB and minocycline, or repeated measures ANOVA using factors of time, followed by Fisher’s least significant difference (LSD) post-hoc testing where appropriate. Non-parametric data were assessed using Kruskal-Wallis to determine overall differences between groups, Friedman’s test to assess changes over time, and Mann-Whitney U post-hoc tests where appropriate. Pearson’s or Spearman’s correlation analysis was carried out to examine correlations between behaviour and inflammatory mediator expression in the brain and spinal cord. The level of significance was set at P < 0.05. All data are presented as the mean ± SEM.
Chapter 5 – Effect of minocycline in OB-SNL

5.3 Results: Study 1

5.3.1 Effect of bulbectomy and chronic minocycline on body weight and fluid intake

Body weight (g) and fluid intake (ml) was measured daily, and here data is presented as values on the day prior to OB/sham surgery, 7 and 14 days post-surgery, and day 1 post-SNL (corresponding to day 18 post-OB surgery) and day 7 and 14 post-SNL. Fluid intake (ml/kg/day) was calculated by \( \frac{\text{volume of fluid intake per day (ml)}}{\text{body weight (kg)}} \). Average drug intake over the study period in minocycline groups is also presented. Repeated measures two-way ANOVA revealed a significant effect of OB (\( F(1,36) = 16.03, P<0.001 \)), time (\( F(5,180) = 1010.2, P<0.001 \)), and a time × OB interaction (\( F(5,180) = 19.18, P<0.001 \)) on body weight (Fig. 5.4A). Post-hoc analysis indicated that there were no differences between groups prior to surgery and all animals gained weight over the study period (Fig. 5.4A). Minocycline did not significantly alter body weight in sham or OB animals. Body weight of OB-water treated rats was significantly lower from day 17 (day of SNL surgery) until the end of the study, and of OB minocycline-treated animals from day 7 post-OB surgery until the end of the study, when compared to sham counterparts.

Repeated measures two-way ANOVA revealed a significant effect of OB (\( F(1,32) = 14.29, P=0.001 \)), minocycline (\( F(1,32) = 32.81, P<0.001 \)), time (\( F(5,160) = 192.10 P<0.001 \)), and time × minocycline interaction (\( F(5,160) = 5.149 P<0.001 \)) on fluid intake over the study period (Fig. 5.4B). OB water-treated animals consumed less water on day 7 and 14 post-SNL compared to sham counterparts, an effect not seen in minocycline-treated animals. Minocycline-treated sham and OB animals consumed less solution from day 14 post-OB until day 14 post-SNL when compared to water-treated controls. Analysis of the amount of minocycline consumed per gram of body weight revealed that both sham and OB animals consumed equivalent amounts of minocycline over the course of the study (Fig. 5.4C).
5.3.1.1 Minocycline levels in brain tissue

At the end of the study (day 38 of treatment), minocycline was detectable (expressed as μg/g of tissue) in cortical brain tissue of minocycline-treated rats using LC - mass spectrometry. Although tissue minocycline levels tended to be higher in OB animals compared to sham counterparts, this effect failed to reach statistical significance (P=0.064, Fig. 5.4D).
Fig 5.4 Effect of OB and minocycline on (A) body weight (B) fluid intake and (C) minocycline intake over the study period (D) Minocycline concentration levels in cortical tissue at the end of the study. *P<0.05, **P<0.01 vs. sham-water, †P<0.05, ‡P<0.01 vs. sham-mino, §P<0.05, ¶P<0.01 vs. OB-water. Data expressed as mean ± SEM, n= 10-12.
5.3.2 Chronic minocycline treatment attenuates OB-induced locomotor hyperactivity

Repeated measures two-way ANOVA revealed a significant effect of OB ($F_{(1,42)} = 15.5, P<0.001$), minocycline ($F_{(1,42)} = 6.46, P=0.015$), time ($F_{(4,168)} = 5.25, P=0.001$) and a time × OB interaction ($F_{(4,168)} = 9.366, P<0.001$) on distance moved per minute over the 5 minute trial period in the open field 14 days post-OB/sham surgery. Post-hoc analysis revealed that OB water-treated rats displayed an increase in distance moved during the first 3 minutes of the test, when compared to sham water-treated controls (Fig. 5.5A). Minocycline did not alter distance moved in sham animals (sham-mino vs. sham-water), but attenuated OB-induced hyperactivity during the 1st, 3rd and 4th minutes.

Analysis of the total distance moved over the 5 minute test revealed a significant effect of OB ($F_{(1,42)} = 15.486, P<0.001$) and minocycline ($F_{(1,42)} = 6.45, P=0.015$) (two-way ANOVA). Post-hoc analysis revealed that OB animals demonstrated a characteristic increase in locomotor activity, compared to their sham counterparts, which was significantly attenuated by chronic minocycline treatment (Fig. 5.5B), indicative of an antidepressant-like effect.

Two-way ANOVA revealed a significant effect of OB on total rearing duration in the open field ($F_{(1,41)} = 8.61, P=0.005$, Fig. 5.5C). Post-hoc analysis revealed that OB water-treated animals displayed increased rearing duration when compared to sham counterparts, an effect not statistically significant in minocycline-treated animals (OB-mino vs. sham-mino). Kruskal-Wallis revealed an overall effect of group on faecal pellet count in the open field ($\chi^2_{(3)} = 12.91, P=0.005$, Fig. 5.5D). Mann-Whitney U post-hoc tests revealed that OB water-treated animals exhibit increased defecation during the test when compared to sham animals, suggesting anxiogenic-like behaviour. Minocycline increased pellet count in sham animals when compared to water-treated animals, but did not significantly alter pellet count in OB animals compared to water-treated counterparts. Neither OB nor minocycline significantly altered time spent in or latency to
enter the inner zone of the open field, although a trend towards less time in the inner zone was observed in OB water-treated animals compared to sham-operated controls (two-way ANOVA effect of OB: $F_{(1,42)} = 3.03$, $P=0.089$).
**Fig. 5.5 Locomotor activity and behaviour in the open field.** (A) Distance moved over one minute time bins (B) Total distance moved (C) Total rearing duration (D) Faecal pellet count (E) time spent in the inner zone (F) Latency to enter the inner zone of the open field *P<0.05, **P<0.01 vs. sham-water, #P<0.05, ##P<0.01, vs. OB-water, ++P<0.01, vs. sham-mino. Data expressed as mean ± SEM, n=10-12.
5.3.3 Effect of chronic minocycline treatment and/or OB on nociceptive responding to mechanical, cold and heat stimuli

Two-way ANOVA revealed a significant effect of OB on 50% mechanical withdrawal thresholds of the hind-paws in the von Frey test 15 days following OB/sham surgery ($F_{(1,41)} = 4.61$, $P=0.038$). Post-hoc analysis revealed that OB minocycline-treated animals exhibited lower mechanical withdrawal thresholds compared to sham counterparts, suggesting mechanical allodynia. Although a similar trend was evident in OB water-treated animals, this failed to reach statistical significance (Fig. 5.6A). Kruskal-Wallis analysis revealed an overall effect of group on the lowest filament force to elicit a response in the von Frey test ($\chi^2_{(3)}=9.2$, $P=0.027$). Mann-Whitney U post-hoc test revealed that OB water-treated animals responded at lower von Frey filaments when compared to sham counterparts, indicative of mechanical allodynia. Minocycline-treated OB animals also responded at lower filament forces when compared to sham counterparts, although this failed to reach statistical significance (Fig. 5.6B).

Two-way ANOVA revealed a significant effect of OB on paw withdrawal latency to application of acetone ($F_{(1,42)} = 4.86$, $P=0.033$) and heat ($F_{(1,42)} = 4.75$, $P=0.035$) following OB surgery. OB water-treated animals displayed lower paw withdrawal latencies to application of acetone (Fig. 5.6C) and heat (Fig. 5.6E) to the hind-paws when compared to sham controls, indicative of OB-induced cold allodynia and thermal hyperalgesia. There were no significant differences between groups for withdrawal frequency to application of acetone to the hind-paw (Fig. 5.6D). Although OB-induced cold allodynia and heat hyperalgesia was not seen in the presence of minocycline treatment, minocycline-treated animals did not exhibit significantly different thresholds compared to water-treated counterparts.
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**Mechanical**

![Bar chart showing mechanical withdrawal thresholds for sham and OB groups with water and minocycline treatments.](chart)

**Cold**

![Bar chart showing cold withdrawal latency for sham and OB groups with water and minocycline treatments.](chart)

**Heat**

![Bar chart showing heat latency to respond to a heat stimulus for sham and OB groups with water and minocycline treatments.](chart)

Fig. 5.6 The effect of OB and minocycline on nociceptive responding to **mechanical**, **cold** and **heat stimuli** (A) 50% mechanical withdrawal thresholds and (B) lowest filament force to elicit a response in the von Frey test. (C) Paw withdrawal latency and (D) withdrawal frequency to application of acetone to the hind-paws. (E) Latency to respond to a noxious heat stimulus in the Hargreaves test. *P<0.05 vs. sham-water, +P<0.01 vs. sham-mino. Data expressed as mean ± SEM, n=10-12.
5.3.4 Chronic minocycline treatment attenuates SNL-induced mechanical allodynia of the ipsilateral hind-paw of both sham and OB animals, with a differential temporal profile

Repeated measures two-way ANOVA revealed a significant effect of minocycline ($F_{(1,35)} = 20.88$, $P<0.001$), time ($F_{(5,175)} = 9.52$, $P<0.001$) and time × minocycline interaction ($F_{(5,175)} = 4.99$, $P<0.001$) on 50% mechanical withdrawal thresholds of the ipsilateral hind-paw following SNL. Post-hoc analysis revealed that SNL decreased thresholds in sham and OB animals at all post-surgery time points when compared to pre-SNL levels, indicating SNL-induced mechanical allodynia (Fig. 5.7A). There was no difference in thresholds between sham and OB water-treated animals following SNL. Minocycline-treated sham-SNL animals exhibited higher thresholds when compared to water-treated controls on day 1, 10 and 14 (Fig. 5.7A). Minocycline-treated OB-SNL animals exhibited increased thresholds compared to water-treated counterparts on day 1, 7, 10 and 14 (Fig. 5.7A). Analysis of the AUC revealed a significant effect of minocycline ($F_{(1,42)} = 18.79$, $P<0.001$). Minocycline increased thresholds in sham-SNL and OB-SNL animals when compared to their water-treated counterparts (Fig. 5.7B), indicating an anti-allodynic effect.

As OB animals exhibited slight mechanical allodynia prior to SNL, mechanical thresholds were expressed as a percentage of pre-SNL values to control for this possible confounding effect. Repeated measures two-way ANOVA revealed a significant effect of minocycline ($F_{(1,35)} = 17.61$, $P<0.001$), time ($F_{(5,175)} = 10.57$, $P<0.001$), time × OB ($F_{(5,175)} = 4.55$, $P=0.001$), time × minocycline ($F_{(5,175)} = 6.38$, $P<0.001$), time × OB × minocycline ($F_{(5,175)} = 3.46$, $P=0.005$) interactions on normalised mechanical withdrawal thresholds of the ipsilateral hind-paw. SNL resulted in decreased thresholds in both sham and OB animals at all post-surgery time points when compared to pre-SNL levels, indicative of SNL-induced mechanical allodynia (Fig. 5.7C). Minocycline-treated sham-SNL animals exhibited increased thresholds on day 10 and 14 when compared to water-treated controls. In comparison, minocycline-treated OB-SNL animals exhibited
increased thresholds on day 1, 7, 10 and 14 when compared to water-treated OB controls, and on day 1 and 7 when compared to sham-SNL minocycline counterparts. Analysis of the AUC revealed a significant effect of minocycline ($F_{(1,40)} = 15.37, P<0.001$) and OB ($F_{(1,40)} = 4.47, P=0.041$, Fig. 5.7D). Minocycline did not significantly alter normalised thresholds in sham-SNL animals. In comparison, minocycline-treated OB-SNL animals exhibited increased thresholds when compared to water-treated OB-SNL and sham-SNL mino-treated controls (Fig. 5.7D), indicating an anti-allodynic effect.

Nociceptive responding of the contralateral hind-paw is presented in the appendix. Minocycline-treated sham animals exhibited increased thresholds of the contralateral hind-paw on day 10 and 14 when compared to water-treated counterparts. OB minocycline-treated animals exhibited increased thresholds on day 10 when compared to water-treated counterparts. When normalised to pre-SNL levels, analysis of AUC revealed that OB minocycline-treated animals exhibited higher thresholds compared to sham controls (Fig. B.6).
Fig. 5.7 Mechanical withdrawal thresholds of the ipsilateral hind-paw following SNL. (A) 50% mechanical withdrawal thresholds. (B) Area under the curve of 50% mechanical withdrawal thresholds. (C) Normalised mechanical withdrawal thresholds (% of pre-SNL values). (D) Area under the curve of normalised mechanical withdrawal thresholds. **P<0.01 vs. sham-SNL-water, ***P<0.01 vs. OB-SNL-water, ++P<0.01 vs. sham-SNL-mino. Data expressed as mean ± SEM. n=10-12.
5.3.5 Chronic minocycline treatment prevents SNL-induced cold allodynia in both sham and OB animals

Repeated measures two-way ANOVA revealed a significant effect of minocycline (F(1,35) = 12.45, P=0.001), OB × minocycline (F(1,35) = 5.07, P=0.031), time (F(1,35) = 52.61, P<0.001), time × OB (F(1,35) = 5.17, P=0.029) and time × minocycline (F(1,35) = 39.77, P<0.001) interactions on the paw withdrawal latency (PWL) to acetone application to the ipsilateral hind-paw prior to and following SNL. Post-hoc analysis revealed that post-SNL, PWL was lower in sham and OB-water treated animals when compared to pre-SNL levels, indicating SNL-induced cold allodynia (Fig. 5.8A). Although there was a trend towards reduced PWL in OB-SNL water-treated animals (sham-SNL 11±1.2s vs. OB-SNL 5.8±1.5s), this was not statistically significant. Minocycline prevented the SNL-induced decrease in PWL in sham and OB animals (Fig. 5.8B), an effect which remained when normalised to pre-SNL levels (Fig. 5.8C), indicating an anti-allodynic effect.

Friedman’s test revealed an effect of time on withdrawal frequency to acetone application to the ipsilateral hind-paw prior to and following SNL (Z = 10.526, P<0.001). Mann-Whitney U post-hoc analysis revealed that post-SNL, withdrawal frequency was higher in sham and OB water-treated animals when compared to pre-SNL levels, indicating SNL-induced cold alldonyia (Fig. 5.8D). OB-SNL water-treated animals displayed significantly enhanced withdrawal frequency compared to sham-SNL controls, indicating exacerbated cold alldonyia (Fig 5.8E), an effect which was not observed when normalised to pre-SNL values (Fig. 5.8F). Minocycline significantly prevented the SNL-induced increase in withdrawal frequency in sham and OB animals (Fig. 5.8E), an effect which remained when normalised to pre-SNL levels (Fig. 5.8F), indicating an anti-nociceptive effect.

Nociceptive responding of the contralateral hind-paw is presented in the appendix. OB water-treated animals exhibited reduced paw withdrawal latency of the contralateral hind-paw following SNL, indicating
contralateral cold allodynia, an effect attenuated by chronic minocycline treatment (Fig. B.7).
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Cold

A

B

C

D

E

F

Fig. 5.8 Nociceptive responding to cold stimuli of the ipsilateral hind-paw prior to and following SNL (A) Paw withdrawal latency to acetone prior to and following SNL (B) Paw withdrawal latency to acetone following SNL (C) Cold thresholds normalised to pre-SNL values (D) Withdrawal frequency to acetone prior to and following SNL. (E) Withdrawal frequency to acetone following SNL (F) Withdrawal frequency normalised to pre-SNL values. δP<0.01 vs. Pre-SNL values, **P<0.01 vs. sham-SNL-water, ##P<0.01 vs. OB-SNL-water. Data expressed as mean ± SEM. n=10-12.
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There was no effect of time, OB or minocycline on latency to respond to a noxious thermal stimulus prior to or following SNL (Fig. 5.9A-C). Nociceptive responding of the contralateral hind-paw is presented in appendix B (Fig. B.7).

**Fig. 5.9** Nociceptive responding to thermal stimuli of the ipsilateral hind-paw prior to and following SNL (A) Paw withdrawal latency to a noxious heat stimulus prior to and following SNL (B) Latency to respond to heat following SNL (C) Heat thresholds normalised to pre-SNL values. Data expressed as mean ± SEM. n=10-12.
5.3.6 Effect of OB and minocycline on open field behaviour following SNL

Animals were re-exposed to the open field 19 days following SNL to examine behavioural effects of OB or minocycline on locomotor activity following peripheral nerve injury. Repeated measures two-way ANOVA revealed a time × OB interaction ($F_{(4,152)} = 2.97, P=0.021$) and an effect of time ($F_{(4,152)} = 18.104, P<0.001$) on distance moved per minute over the 5 minute trial period in the open field following SNL. There was no difference in distance moved between OB-SNL and sham-SNL water-treated animals. OB-SNL minocycline-treated rats displayed reduced distance moved when compared to sham-SNL minocycline-treated rats during the last minute of the test only (Fig. 5.10A). There were no significant effects of OB or minocycline on total distance moved or rearing duration in the open field following SNL (Fig. 5.10 B&C). Kruskal-Wallis test revealed an overall effect of group for faecal pellet count in the open field following SNL ($\chi^2_{(3)} = 8.26, P=0.041$, Fig. 5.10D). Mann-Whitney U post-hoc test revealed that OB-SNL water-treated animals exhibited increased defecation during the test compared to sham-SNL controls, an effect not significantly altered by minocycline treatment. Two-way ANOVA revealed an effect of OB on time spent in the inner zone ($F_{(1,38)} = 7.65, P=0.009$). OB-SNL water-treated animals spent less time spent in the inner zone when compared to sham-SNL controls, indicative of anxiety-like behaviour, an effect not altered by minocycline treatment (Fig. 5.10E). Neither OB nor minocycline significantly altered latency to enter the inner zone of the open field (Fig. 5.10F).
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Fig. 5.10 Locomotor activity and behaviour in the open field following SNL. (A) Locomotor activity in the open field (B) Total distance moved (C) Rearing duration (D) Faecal pellet count (E) Time in the inner zone of the open field (F) Latency to enter the inner zone of the open field. **P<0.01 vs. sham-SNL-water, ++P<0.01 vs. sham-SNL-mino. Data expressed as mean ± SEM. n=10-12.
5.3.7 Effect of OB and/or chronic minocycline treatment on the mRNA expression of neuroinflammatory markers in the dorsal horn of the spinal cord following SNL

Inflammatory marker mRNA expression in the dorsal horn of the lumbar enlargement of the spinal cord on day 20 post-SNL was determined by qRT-PCR and data were expressed as a percentage of sham-SNL water-treated controls. Two-way ANOVA revealed an effect of OB (F(1,33) = 8.20, P=0.007) on IL-1β mRNA; an effect of minocycline on IL-1β (F(1,33) = 5.11, P=0.031) and IL-6 (F(1,35) = 4.83, P=0.034) mRNA; and an OB × minocycline interaction on IL-10 (F(1,18) = 4.72, P=0.043) and SOCS3 (F(1,21) = 5.79, P=0.025) mRNA in the ipsilateral dorsal horn. Post-hoc analysis revealed that OB-SNL water-treated animals exhibited less IL-1β mRNA in the dorsal horn when compared to sham-SNL water-treated controls (Fig. 5.11C). Minocycline-treated sham-SNL animals exhibited decreased IL-1β and increased IL-6, IL-10 and SOCS3 mRNA when compared to water-treated controls, effects not observed in OB-SNL minocycline-treated animals (Fig. 5.11 C, E-G). Neither OB nor minocycline significantly altered CD11b, GFAP, TNFα or IL-1ra mRNA in the ipsilateral dorsal horn in either sham or OB animals following SNL.

Expression of these mediators in the contralateral dorsal horn is presented in the appendix (Fig. B.8).
Fig. 5.11 Expression of neuroinflammatory mediators in the ipsilateral lumbar enlargement of the dorsal horn of sham and OB animals following SNL. (A) CD11b (B) GFAP (C) IL-1β (D) TNFα (E) IL-6 (F) IL-10 (G) SOCS3 (H) IL-1ra mRNA expression in the dorsal horn. * P<0.05, **P<0.01 vs. sham-SNL-water, +++P<0.01 vs. sham-SNL-mino. Data expressed as mean ± SEM. N=10-11.
5.3.8 Effect of OB and/or chronic minocycline treatment on the expression of neuroinflammatory markers in the PFC following SNL

Analysis revealed that the expression of inflammatory mediators in the brain did not differ between ipsilateral or contralateral sides, thus data were pooled for subsequent analysis. Two-way ANOVA revealed an effect of OB on GFAP \((F_{(1,36)} = 6.39, P=0.016)\) and IL-10 mRNA \((F_{(1,34)} = 7.89, P=0.01)\), an effect of minocycline on IL-10 mRNA \((F_{(1,34)} = 9.73, P=0.004)\), and an OB × minocycline interaction on CD11b \((F_{(1,35)} = 4.22, P=0.047)\), IL-1β \((F_{(1,37)} = 12.47, P=0.001)\), IL-6 \((F_{(1,34)} = 5.53, P=0.025)\) and SOCS3 \((F_{(1,37)} = 8.52, P=0.006)\) mRNA in the PFC following SNL. TNFα or IL-1ra mRNA expression was not significantly altered by OB or minocycline treatment.

Post-hoc analysis revealed that OB-SNL animals exhibited increased GFAP mRNA when compared to sham-SNL controls (Fig. 5.12B). Minocycline-treated sham-SNL animals exhibited decreased CD11b, IL-1β and SOCS3 mRNA when compared to water-treated controls (sham-SNL-mino vs. sham-SNL-water), effects not seen in minocycline-treated OB-SNL animals (OB-SNL-mino vs. OB-SNL-water) (Fig. 5.12 A, C, G). In comparison, minocycline-treated OB-SNL animals exhibited augmented IL-1β and IL-10 mRNA when compared to water-treated OB-SNL and sham-SNL minocycline counterparts, and increased IL-6 mRNA when compared to water-treated OB-SNL counterparts (Fig. 5.12 C, E, F).
Fig. 5.12 Expression of neuroinflammatory mediators in the PFC of sham and OB animals following SNL. (A) CD11b (B) GFAP (C) IL-1β (D) TNFα (E) IL-6 (F) IL-10 (G) SOCS3 (H) IL-1ra mRNA expression in the PFC. * P<0.05 vs. sham-SNL-water, **P<0.01 vs. OB-SNL-Water, ***P<0.01 vs. sham-SNL-mino. Data expressed as mean ± SEM. N=10-11.
5.3.9 Effect of OB and/or chronic minocycline treatment on the expression of neuroinflammatory markers in the amygdala following SNL

Two-way ANOVA revealed an OB × minocycline interaction on CD11b ($F_{(1,36)} = 7.61, P=0.009$), an effect of OB on GFAP ($F_{(1,36)} = 13.44, P=0.001$) and an effect of minocycline on TNFα ($F_{(1,35)} = 5.70, P=0.023$) mRNA levels in the amygdala following SNL. *Post-hoc* analysis revealed that OB-SNL water-treated animals did not exhibit any alterations in the mRNA expression of CD11b or GFAP when compared to sham-SNL controls (Fig. 5.13). Minocycline treatment did not alter the mRNA expression of CD11b or TNFα in sham-SNL animals when compared to water-treated controls. In comparison, minocycline-treated OB-SNL animals exhibited increased CD11b and GFAP mRNA when compared to sham-SNL minocycline counterparts (OB-SNL-mino vs. sham-SNL-mino, Fig. 5.13 A&B). In addition, minocycline-treated OB-SNL animals exhibited increased CD11b and lower TNFα mRNA when compared to water-treated controls (OB-SNL-mino vs. OB-SNL water, Fig. 5.13D).

There were no significant correlations between behaviour and mRNA expression in the PFC, amygdala or spinal cord.
Fig. 5.13 Expression of neuroinflammatory mediators in the amygdala of sham and OB animals following SNL. (A) CD11b (B) GFAP (C) IL-1β (D) TNFα (E) IL-6 (F) IL-10 (G) SOCS3 (H) IL-1ra mRNA expression. #P<0.05 vs. OB-SNL-water, ++P<0.01 vs. sham-SNL-mino. Data expressed as mean ± SEM. N=10-11.
5.4 Study 2

The effect of acute minocycline on depressive-like behaviour and nociceptive responding to mechanical stimuli in the OB rat, prior to and following peripheral nerve injury

5.4.1 Acute minocycline treatment does not alter OB-induced hyperactivity

Repeated measures two-way ANOVA revealed an effect of OB ($F_{(1,29)} = 10.85, P=0.003$), time ($F_{(4,116)} = 10.99, P=0.01$) and a time × OB interaction ($F_{(4,116)} = 4.98, P=0.001$) on distance moved per minute over the 5 minute trial period in the open field 14 days post-OB/sham surgery. Post-hoc analysis revealed that OB animals exhibited increased distance moved during the first minute of the test, when compared to sham controls, an effect not altered by minocycline treatment (Fig. 5.14A).

Two-way ANOVA revealed an effect of OB on total distance moved in the open field ($F_{(1,29)} = 10.85, P=0.003$). OB vehicle-treated animals demonstrated increased locomotor activity compared to sham counterparts, an effect not altered by acute minocycline treatment (Fig. 5.13B). Time spent in the inner zone was not significantly altered by OB or minocycline alone, however an OB × mino interaction approached significance ($F_{(1,28)} = 3.51, P=0.077$, Fig. 5.14C). Kruskal-Wallis revealed an overall effect of group for pellet count ($\chi^2_{(3)} = 11.31, P=0.01$). OB animals exhibited increased defecation during the test when compared to sham controls, an effect prevented by minocycline treatment (Fig. 5.14D).

Two-way ANOVA revealed an effect of minocycline on total time spent rearing ($F_{(1,29)} = 5.23, P=0.03$) and grooming ($F_{(1,27)} = 6.76, P=0.015$) in the open field. Post-hoc analysis revealed that rearing duration was significantly reduced in minocycline-treated OB animals when compared to vehicle-treated controls (Fig. 5.14E). Minocycline-treated sham and OB animals exhibited reduced grooming duration (Fig. 5.14F).
Fig. 5.14 Locomotor activity in the open field on day 14 post sham/OB surgery (A) Distance moved in the open field in 5 minute time bins (B) Total distance moved in the open field (C) Time in the inner zone of the open field (D) Faecal pellet count (E) Rearing duration (F) Grooming duration. Data presented as mean ± SEM, n = 7-10. *P<0.05, **P<0.01 vs. sham-veh, †P<0.05, ‡P<0.01 vs. sham-mino, ††P<0.05, ‡‡P<0.01 vs. OB-veh, †††P<0.05 effect of minocycline (ANOVA).
5.4.2 Acute minocycline treatment does not significantly alter OB-induced mechanical allodynia

Two-way ANOVA revealed that there was no effect of acute minocycline treatment on 50% mechanical withdrawal thresholds of the hind-paw when examined 14 days following OB/sham surgery (Fig. 5.15A). However, OB animals demonstrated a tendency to exhibit reduced mechanical withdrawal thresholds when compared to shams ($F_{(1,27)} = 3.22$, $P=0.084$).

Kruskal-Wallis analysis of the lowest filament force to elicit a response revealed an overall effect of group ($\chi^2_{(3)} = 11.72$, $P=0.008$, Fig. 5.15B). Mann-Whitney U post-hoc tests revealed that OB vehicle-treated animals responded at lower filaments in the von Frey test when compared to sham counterparts, indicating mechanical allodynia, an effect which just failed to reach significance in minocycline-treated OB animals (Fig. 5.15B).

![Fig. 5.15](image.png)

**Fig. 5.15 Mechanical withdrawal thresholds of the hind-paws of sham and OB rats.**(A) 50% mechanical withdrawal thresholds 14 days after sham/OB surgery (B) Lowest filament force to elicit a response 14 days after sham/OB surgery. **$P<0.01$ vs. Sham-Veh controls. Data presented as Mean ± SEM, $n = 7-10$**
5.4.3 Acute minocycline treatment prevents SNL-induced mechanical allodynia of the ipsilateral hind-paw of sham and OB animals

Repeated measures two-way ANOVA revealed a significant time × minocycline interaction ($F_{(1,26)} = 12.84, P=0.001$) on mechanical withdrawal thresholds of the ipsilateral hind-paw prior to and following SNL surgery. SNL resulted in lower mechanical withdrawal thresholds in sham and OB animals when compared to pre-SNL values, indicating SNL-induced mechanical allodynia (Fig. 5.16A). Acute minocycline pretreatment prevented SNL-induced allodynia in sham and OB animals on day 1 post-surgery (Fig. 5.16B), an effect which remained following normalisation to pre-SNL values (Fig. 5.16 C&D). Neither OB nor minocycline altered withdrawal thresholds of the contralateral hind-paw (see appendix Fig. B.9).
Fig. 5.16 Mechanical withdrawal thresholds of the ipsilateral hind-paw of sham and OB rats prior to and following SNL. (A) 50% mechanical withdrawal thresholds from pre-SNL to 1 day after SNL surgery (B) Effect of OB and minocycline on 50% mechanical withdrawal thresholds on 1 day after SNL surgery (C) and (D) Normalised withdrawal thresholds (percentage of pre-SNL values). *P<0.05 sham and OB vs. pre-SNL values, *P<0.05, **P<0.01 vs. sham-SNL-vehicle, #P<0.05 vs. OB-SNL-vehicle. Data presented as Mean ± SEM, n = 7-10.
5.4.4 Comparison of the effect of chronic and acute minocycline treatment on the development of SNL-induced mechanical allodynia on day 1 post-surgery

**Fig. 5.17** The effect of chronic and acute minocycline treatment on mechanical withdrawal thresholds (absolute and normalised) in the ipsilateral hind-paw prior to and following SNL. (A) and (B) Chronic minocycline treatment prevents the development of SNL-induced mechanical allodynia in OB, but not sham animals, on day 1 post-surgery. (C) and (D) Acute minocycline pretreatment prevents the development of SNL-induced mechanical allodynia on day 1 post-surgery in both sham and OB animals. *P<0.05 sham and OB vs. pre-SNL values, *P<0.05, **P<0.01 vs. sham-SNL-vehicle/water, #P<0.05 vs. OB-SNL-vehicle/water, ++P<0.01 vs. sham-SNL-mino. (n=10-11 chronic, n=7-10 acute).
5.5 Discussion

In brief, the results presented in the current chapter show that chronic, but not acute, minocycline treatment attenuated OB-induced hyperactivity in the open field, indicative of an antidepressant-like effect. Neither acute nor chronic minocycline significantly altered nociceptive responding to mechanical stimuli in either sham or OB rats prior to peripheral nerve injury. SNL resulted in characteristic mechanical and cold allodynia of the ipsilateral hind-paw of sham and OB animals. OB animals exhibited enhanced SNL-induced cold alldynia when compared to sham counterparts. Chronic minocycline treatment attenuated SNL-induced mechanical allodynia in OB rats at almost all time-points over the 2-week testing period, an effect only observed from day 10 post-SNL surgery in sham rats. SNL-induced cold alldynia was attenuated by chronic minocycline in both sham and OB rats. Acute minocycline prevented the development of SNL-induced mechanical alldynia on day 1 post-surgery to a comparable degree in sham and OB animals, whereas chronic minocycline prevented the development of SNL-induced mechanical alldynia on day 1 post-surgery in OB animals only. OB-SNL animals exhibited lower IL-1β in the ipsilateral spinal cord and increased GFAP in the PFC when compared to sham-SNL counterparts. Chronic minocycline elicited an anti-inflammatory effect in the spinal cord (reduced IL-1β, and increased IL-10, IL-6 and SOCS3 mRNA) and PFC (reduced CD11b and IL-1β mRNA) of sham-SNL animals, effects not observed in OB-SNL animals. In contrast, IL-1β, IL-6 and IL-10 mRNA in the PFC was increased, while CD11b was increased and TNFα decreased in the amygdala of minocycline-treated OB-SNL animals, when compared to water-treated counterparts. Thus, the current data demonstrated that chronic inhibition of microglial activation elicits an antidepressant-like effect and protects against the development of neuropathic pain following peripheral nerve injury in the OB model, effects accompanied by altered expression of inflammatory mediators at spinal and supraspinal sites responsible for the regulation of pain and emotion.
5.5.1 Inhibition of microglial activation – a new treatment strategy for depression

While it is well known that minocycline is capable of inhibiting microglial activation both in vitro and in vivo, it was unknown if this compound would accumulate in the brain in disease pathologies such as depression. As such, this study assessed cortical minocycline levels using mass spectrometry. Minocycline administered chronically through drinking water reached brain tissue and accumulated in levels that are in accordance with previous reports following systemic administration (Colovic and Caccia, 2003). Cortical minocycline levels appeared to be slightly higher in OB animals which may be due to blood brain barrier dysfunction observed in OB animals (Wrynn et al., 2000a). Thus, minocycline may have had a greater effect on microglial inhibition in OB vs. sham rats.

Correlating with a wealth of previous data (Chapter 3&4, Roche et al., 2007, Song and Leonard, 2005), OB animals exhibited characteristic hyperactivity in the open field. This OB-induced locomotor response to a novel environment was prevented by chronic, but not acute, minocycline treatment, confirming the antidepressant-like effect of this drug in the OB model. Similarly, a recent study has also demonstrated that repeated administration of minocycline 50mg/kg i.p. for 4 weeks attenuated OB-induced hyperactivity in the open field (Borre et al., 2012b). Minocycline has been reported to suppress olfactory neuron death after unilateral olfactory bulbectomy (Kern et al., 2004), attenuate traumatic brain injury (TBI)-induced olfactory bulb atrophy and cognitive decline (Siopi et al., 2012a, Siopi et al., 2012b), and block TBI-induced microglial activation and locomotor hyperactivity (Homsi et al., 2010). Thus, microglial activation may be involved in the development of the OB syndrome, an effect attenuated by chronic/repeated minocycline administration. Neurodegeneration following bulbectomy has been described in regions such as the piriform cortex, hippocampus, amygdala, dorsal raphe nucleus and locus coeruleus (Norrholm and Ouimet, 2001, Nesterova et al., 1997, Capurso et al., 1997, Leung and Wilson, 2003, Koliatsos et al., 2004) and exaggerated

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or prolonged inflammatory responses from microglia are a key component of neurodegenerative processes (Polazzi and Contestabile, 2002). Reactive microgliosis occurs in response to neuronal damage, resulting in destruction of adjacent neurons, which causes further microglial activation and a propagating cycle of inflammation and impairment of neuronal integrity (Block and Hong, 2005). Region-specific neuroinflammation may impact on cortico-limbic circuits and lead to psychiatric disorders (Steiner et al., 2011, Schmitt et al., 2011). It is likely that bulbectomy-induced neuronal damage activates microglia resulting in neuroinflammation which further promotes neurodegeneration of the anterograde and retrograde neural connections from the bulbs, causing the behavioural deficits observed in the model. OB-induced increased expression of IL-1β and COX-2 in the hippocampus has been shown to occur at the time of onset of locomotor hyperactivity, and increases in microglial CD68 expression parallels passive avoidance deficits (Borre et al., 2012b, Borre et al., 2012a). Minocycline has been shown to prevent retrograde axonal degeneration in CNS injury (Stirling et al., 2004), thus administration of minocycline prior to and immediately following OB surgery as in the current study and in that of Borre and colleagues (2012b), may prevent microglial activation and neuronal re-organisation that occurs in response to removal of the bulbs and therefore mitigate some of the pathological alterations seen in the model. In order to address if a similar effect would occur when microglia were inhibited following the development of the OB syndrome, minocycline was administered once off on day 14 post-bulbectomy, 3h prior to open field exposure (study 2). In this instance, minocycline failed to attenuate OB-induced hyperactivity, apart from a strong trend the last minute. Effects observed during the last minute may relate to anxiolytic-like effects of minocycline administration. Mar et al. reported that lorazepam (an anxiolytic) reduced OB-related hyperactivity by decreasing the activity towards the end of a 5 minute open field trial (Mar et al., 2002). In addition, the current study demonstrated that acute minocycline tended to increase time in the inner zone and significantly reduced defecation and rearing behaviour in OB animals. Therefore, acute minocycline administration appears to exert an anxiolytic-like effect in OB
animals, whereas chronic administration of minocycline appears to be required to elicit an antidepressant-like effect in the model. Anxiolytic-like effects of minocycline have been reported in other models. Intracerebroventricular infusion of minocycline reduced anxiety-like behaviour in the open field in mice pre-exposed to chronic stress and then subjected to cardiac arrest-cardiopulmonary resuscitation, a model associated with hippocampal neuroinflammation (Neigh et al., 2009). In addition, minocycline attenuated traumatic brain injury-induced anxiety-like behaviour in the elevated plus maze (Fan et al., 2006). Taken together, these data indicate that modulation of microglial activity using minocycline can attenuate depressive- and anxiety-like behaviours in models of central injury.

5.5.2 Inhibiting microglial activation does not alter nociceptive responding to mechanical or thermal stimuli

In accordance with previous data (Chapter 3 and Burke et al., 2010), OB animals exhibited mechanical and cold allodynia. While we did not observe altered responses to heat stimuli in previous chapters, this study demonstrated that OB animals also exhibited slight thermal hyperalgesia. Previous reported data from our laboratory has demonstrated transient thermal hyperalgesia in OB animals when assessed using the hot plate test (Burke et al., 2010) and thermal hyperalgesia has been reported in the tail flick test (Rodriguez-Gaztelumendi et al., 2006). Conversely, OB animals have exhibited thermal hypoalgesia to radiant heat applied to the hind-paw (Wang et al., 2010b, Su et al., 2010). It is possible that the effect observed in the current study may be due to the earlier time-point at which thermal hyperalgesia was assessed (Chapter 3 – day 18, Chapter 4 – day 23, Chapter 5 – day 16). Minocycline treatment did not significantly alter nociceptive thresholds to mechanical (acute/chronic), cold or heat stimuli in sham animals, correlating with previous reports when administered acutely (Mika et al., 2009, Bastos et al., 2007), repeatedly (3 days) (Yoon et al., 2012) or chronically (Pu et al., 2012). However, chronic treatment did appear to slightly reduce thresholds (lowest filament response, cold and heat response...
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latencies) in sham animals, an effect which resulted in the OB-associated allodynia not reaching significance in minocycline-treated animals. Nonetheless, minocycline did not significantly alter OB-associated allodynia (OB-water vs. OB-mino), indicating a dissociation between depressive-like behaviour and allodynia prior to nerve injury, in accordance with Chapter 4.

5.5.3 Inhibition of microglial activation attenuates allodynia induced by peripheral nerve injury – enhanced effects in the presence of a depressive-like phenotype

In accordance with previous chapters (3&4) and the literature (Chung et al., 2004, Kim and Chung, 1992, Moriarty et al., 2012), SNL resulted in mechanical and cold allodynia of the ipsilateral hind-paw of sham and OB animals. While mechanical allodynia did not differ between sham and OB animals following SNL surgery, SNL-induced cold allodynia was enhanced in OB animals similar to that observed in Chapter 3. Cold allodynia is assessed by 2 different means in our studies and it should be noted that while in the current study OB animals exhibited increased number of responses to acetone application, in Chapter 3 OB animals demonstrated reduced paw withdrawal latencies to a cold stimulus. The discrepancy between chapters may be due to differential subjective ratings between experimenters in these two studies. Furthermore, these data together with the pre-SNL allodynia which was also observed in Chapter 3 and the present chapter indicates that the stress of repeated i.p. injections most likely accounted for the lack of effect of OB in the AMI study (Chapter 4). Thus, the current data replicate the findings from Chapter 3 and suggest that exacerbated SNL-induced cold allodynia in OB animals is reproducible under conditions of minimal stress.

Minocycline pretreatment attenuated mechanical allodynia in sham animals following spinal nerve ligation, as previously reported (Guasti et al., 2009, Lin et al., 2007, Pu et al., 2012, Mei et al., 2013, Zhang et al., 2012, Mei et al., 2011). However, the development of the anti-nociceptive effect of minocycline was markedly different between the chronic and acute
treatment regimes used in the current chapter. With chronic treatment, sham-SNL animals developed mechanical allodynia from day 1 - 7, however by day 10 minocycline treatment significantly increased mechanical withdrawal thresholds, an effect which was sustained until day 14. Moreover, the development of cold allodynia was significantly prevented at this time point. In contrast, acute pretreatment with minocycline prior to surgery successfully prevented the development of mechanical allodynia on day 1 post-surgery in sham-SNL animals. To our knowledge, minocycline has not previously been administered in drinking water in a model of neuropathic pain, and it is possible that minocycline delivered by this route of administration does not reach levels sufficient to inhibit microglial activation in the days following nerve injury. The lack of effect of minocycline on mechanical allodynia in the first few days after SNL surgery in sham animals has been previously observed following SNL using 30mg/kg i.p for 14 days (Guasti et al., 2009) and intrathecally (2-6μg/h for 7 days) (Lin et al., 2007). However, higher intrathecal doses (60-80μg/day) administered chronically prevented the development of mechanical alldonyia from day 1 (Zhang et al., 2012, Pu et al., 2012). It is possible that the large bolus of minocycline used in the acute study (80mg/kg) resulted in higher levels at the spinal cord prior to surgery which resulted in prevention of alldonyia on day 1, whereas in the chronic study, the levels may have been distributed differentially throughout the day.

Minocycline pretreatment also attenuated SNL-induced mechanical allodynia in OB animals. However, the temporal profile of the attenuation of mechanical allodynia by minocycline was markedly different in sham vs. OB animals. Chronic minocycline attenuated mechanical alldonyia in OB animals at almost all time points (except day 3), an effect only observed from day 10 post-SNL surgery in sham rats. Furthermore, when examined as a percentage of pre-SNL, minocycline resulted in a greater relative effect of attenuating mechanical alldonyia in OB animals, and also increased thresholds in the contralateral hind-paw. Furthermore, chronic minocycline treatment attenuated bilateral cold alldonyia exhibited in OB animals. Acute
pretreatment with minocycline prior to surgery successfully prevented the development of mechanical allodynia on day 1 post-SNL in OB animals, to a comparable degree as observed in sham animals.

The earlier onset of action of chronic minocycline treatment in attenuating SNL-induced mechanical allodynia OB rats may potentially relate to the higher levels of minocycline which accumulated in the brains of OB vs. sham rats. Alternatively, minocycline may be more effective in the presence of a preceding depressive-like phenotype, possibly due to enhanced responsivity of the immune system in depression. Minocycline treatment may also affect distinct glia phenotypes in a different manner in sham and OB animals. During CNS injury, microglial activation is divided into two phenotypic profiles: the classical M1 and the alternative M2 activation state (Michelucci et al., 2009, Colton, 2009). The classical M1 pathway is pro-inflammatory, producing IL-1β, TNF-α, IL-6 and reactive oxygen- and nitrogen species (Colton, 2009). The alternative M2 phenotype is believed to be neuroprotective, and is important when switching from a pro-inflammatory to an anti-inflammatory profile, thus limiting damage (Colton, 2009). Following spinal cord injury, M1 polarisation is favoured, with a early and transient M2 response (Kigerl et al., 2009, David and Kroner, 2011). Minocycline selectively inhibits M1 polarization of microglia by attenuating the induction of the expression of M1 microglia markers during the progressive phase in a mouse model of amyotrophic lateral sclerosis, but does not alter the transient increase in expression of M2 microglia markers during the early phase of the disease (Kobayashi et al., 2013). Although speculative, the effect of minocycline on microglial phenotypes may be divergent between sham and OB animals following nerve injury, as OB animals have shown enhanced circulating IL-1β (Song et al., 2009) which suggests M1 polarisation, and as such may have more microglia of the M1 phenotype on which minocycline can exert a greater effect. In addition, it is likely that nerve injury induces different effects in sham and OB animals due to neuronal, endocrine, neurochemical and immune alterations in the OB rat that may influence the physiological response to nerve injury when
compared to sham animals. Interaction of minocycline with these may be responsible for the differential effects seen here.

The lack of effect of minocycline in OB’s on day 3 post-injury is interesting. This may reflect peak activation of glia at this time-point, which minocycline treatment is unable to overcome. In accordance with this hypothesis, a substantial increase in the number and intensity of p38-immunoreactive microglia occurs 3 days after SNL in the ipsilateral L5 spinal cord and DRG (Jin et al., 2003). p38 mitogen-activated protein kinase regulates the expression of cytokines, COX-2, and iNOS via transcriptional, post-transcriptional (mRNA stability) and translational regulation (Widmann et al., 1999, Ji and Woolf, 2001, Koistinaho and Koistinaho, 2002), all of which are increased 3 days after SNL (O’Rielly and Loomis, 2006). OB animals may exhibit a heightened cascade of neuroinflammation which may not be attenuated to the same degree as at other time points in these animals, and as such SNL-induced mechanical allodynia is expressed at this point. However, later on, pro-inflammatory cytokines may be reduced somewhat and anti-inflammatory cytokines increased, thus allowing for the effect of minocycline recover.

Taken together, the above data indicate that minocycline successfully attenuates SNL-induced mechanical and cold allodynia in both sham and OB rats, although the temporal profile of anti-allodynic effects differs. Such data may indicate that treatment of depression with microglial inhibitors may protect against the development of neuropathic pain following nerve injury.

5.5.4 Effect of chronic minocycline on inflammatory mediators in the spinal cord, PFC and amygdala

The anti-inflammatory effects of minocycline are well established (Fan et al., 2005, Giuliani et al., 2005, Henry et al., 2008a, Hua et al., 2005, Krady et al., 2005, Leite et al., 2011, Yrjanheikki et al., 1999, Zanjani et al., 2006, Yoon et al., 2012). The current study reports that chronic minocycline
Chapter 5 – Effect of minocycline in OB-SNL

treatment has a classical anti-inflammatory effect in the spinal cord and PFC of sham-SNL animals, with a differential mRNA expression profile observed in OB rats.

In the spinal cord, the mRNA expression of markers of microglial (CD11b) and astrocyte (GFAP) activation were upregulated following SNL as previously shown (Tanga et al., 2004) in the ipsilateral dorsal horn of both sham and OB animals when compared to the contralateral side when measured 20 days post-surgery (comparisons not shown). CD11b or GFAP mRNA expression in the ipsilateral dorsal horn was not altered by minocycline at the time point measured. However, it cannot be ruled out that microglial morphology or function may have been altered, or that minocycline altered microglial CD11b mRNA expression at an earlier time point. Nonetheless, chronic minocycline treatment elicited an anti-inflammatory effect in the spinal cord of sham-SNL animals by reducing the expression of the pro-inflammatory cytokine IL-1β while increasing the expression the anti-inflammatory cytokine IL-10 and also SOCS3 in the spinal cord. IL-10 induces the expression of SOCS3 (suppressor of cytokine signalling 3), and binding of IL-6 to its receptor also induces SOCS3 expression, which results in an anti-inflammatory effect. The anti-inflammatory effect of minocycline has been well described at the level of the spinal cord. For example, minocycline has been shown to reduce HIV-1 gp120- (Ledeboer et al., 2005) and LPS- (Yoon et al., 2012) induced IL-1β mRNA expression in the spinal cord, effects associated with the attenuation of mechanical allodynia and thermal hyperalgesia. Moreover, intrathecal minocycline prevents IL-1β-induced thermal hyperalgesia (Sung et al., 2012). Repeated minocycline treatment has been shown to increase spinal mRNA expression of IL-10 following spinal cord injury (Lee et al., 2003). Moreover, minocycline has been shown to prevent spinal cord microglial activation and allodynia/hyperalgesia in a number of studies in models of inflammatory nociception (Li et al., 2010, Hua et al., 2005), chronic pancreatitis (Liu et al., 2012), spinal cord injury (Hains and Waxman, 2006), and spinal nerve ligation (Lin et al., 2007). Thus, the anti-inflammatory effects of minocycline are associated with attenuation of
enhanced pain behaviour, most likely accounting for at least some of its effect in reducing SNL-induced mechanical and cold allodynia in sham animals in the current chapter. In addition, minocycline resulted in an anti-inflammatory effect in the PFC, but not amygdala, of sham-SNL animals by reducing mRNA expression of CD11b and IL-1β. Systemic minocycline has been shown to reduce mRNA levels of IL-1β and IL-6 in the cortex following LPS (Henry et al., 2008a). In models of peripheral nerve injury, pain-related increases in the mRNA and protein levels of TNFα, IL-1β, IL-6 and/or NFκB have been reported in the brain and PFC and are attenuated by cytokine inhibitors (pentoxifylline), corticosteroids, and a PPAR-gamma agonist (pioglitazone) with concurrent reductions in neuropathic pain behaviour (Xie et al., 2006, Liu et al., 2007, Jia et al., 2010, Apkarian et al., 2006). Thus, inhibition of neuropathic pain-related neuroinflammation in the PFC may result in reduction of allodynia and/or hyperalgesia.

Direct comparison of OB-SNL and Sham-SNL on inflammatory mediator expression within the spinal cord, PFC and amygdala revealed that OB-SNL water-treated animals exhibited reduced IL-1β in the spinal cord and increased GFAP in the PFC, however, none of the other markers were altered. Increased GFAP in the PFC of OB-SNL animals is in accordance with data from Chapter 3 and 4, thus re-confirming that OB-SNL animals exhibit robust and sustained astrocyte activation in the PFC, the precise functional significance of which remains to be determined.

The effect of chronic minocycline treatment on cytokine expression in the CNS was strikingly different in OB-SNL animals. Specifically, the anti-inflammatory profile exerted by minocycline in sham-SNL animals was not seen in the spinal cord or PFC of OB-SNL animals. Although it cannot be ruled out that minocycline altered inflammatory mediator mRNA expression in the spinal cord at earlier time points, it may suggest that minocycline has a distinct site of action in OB animals. Minocycline pretreatment has been shown to attenuate LPS-induced brain IL-1β but have no effect on plasma IL-1β level, suggesting that minocycline can have anti-inflammatory properties within the brain that are independent of alterations in the
periphery (Henry et al., 2008a). Chronic minocycline resulted in increased IL-10, IL-1β, and IL-6 in the PFC, and increased CD11b with a concurrent decrease in TNFα in the amygdala of OB-SNL animals. Thus, minocycline has a differential effect on the expression of spinal and supraspinal inflammatory mediators in sham and OB animals. Increased levels of glial marker expression at the late post-surgery time-point examined in OB-SNL minocycline-treated animals may indicate enhanced glial function or a phenotypic switch. IL-10 has been shown to induce polarisation to M2 microglia (Michelucci et al., 2009) and IL-10 levels are enhanced in OB-SNL animals by minocycline in the current study. This could be a beneficial mechanism as microglia are important in phagocytosis, dampening inflammation and increasing release of growth factors which are critical for repair and regeneration (Loane and Byrnes, 2010). These data suggest a more complex role for minocycline in the presence of a depressive-like phenotype. Although several studies have demonstrated an anti-inflammatory effect of minocycline as discussed, one study has reported that chronic minocycline treatment (10mg/kg i.p. for 10 days) resulted in increased IL-1β, IL-6 and TNFα mRNA in the brains of mice (Wisor et al., 2011). Taken together, these data indicate bidirectional modulation of inflammatory gene expression by minocycline, depending on the experimental condition. In addition to enhancing IL-10 mRNA, the expression of IL-1β was also enhanced in the PFC of OB-SNL minocycline-treated animals. Although IL-10 is more likely to reduce IL-1β expression due to its anti-inflammatory effects, it has been shown that microglial cells treated with IL-10 exhibited an increase of IL-1β expression levels (Michelucci et al., 2009), an effect which may relate to different concentrations of this cytokine. In addition to the well-known pro-inflammatory effect of IL-1β, it also plays a role in the repair of the CNS via the induction of astrocyte and microglia derived IGF-1 (Mason et al., 2001), and using cultured explants of sciatic nerve, IL-1β from activated macrophages increased nerve growth factor following injury (Lindholm et al., 1987). Therefore, cytokines and glia can play a dual role in the
resolution of neuronal injury, effects which may be mediated differentially by minocycline in the presence or absence of bulbectomy.

5.5.5 Conclusion

The data presented in the current chapter indicate that chronic minocycline treatment prevents the development of depressive-like behaviour in the OB rat, and attenuates SNL-induced mechanical and cold allodynia in the model. In addition, chronic minocycline treatment differentially modulates the expression genes encoding inflammatory mediators in sham and OB animals following SNL.

These data further support a role for microglia in the pathogenesis of depression, the development of neuropathic pain, and the interaction between these disorders.

5.5.6 Summary of aims and subsequent findings

(1) Determined the effect of acute and chronic inhibition of microglial activation on depressive-like behaviour in the OB rat

(i) Chronic, but not acute, minocycline treatment elicits an antidepressant-like effect in the OB rat

(2) Examined the effect of acute and chronic minocycline administration on nociceptive responding to mechanical and thermal stimuli

(ii) Neither acute nor chronic altered nociceptive responding in sham or OB

(3) Investigated the effect of chronic minocycline on SNL-induced mechanical and cold allodynia, and examined if altered differentially in sham vs. OB rats

(iii) Differential effects of chronic minocycline treatment of prevention of SNL-induced mechanical allodynia, similar effects on prevention of cold
allodynia. Acute minocycline prevented the development of SNL-induced mechanical allodynia to an equal degree in sham and OB.

(4) Evaluated the effect of chronic minocycline on the mRNA expression of genes encoding inflammatory mediators in the PFC, amygdala and spinal cord following SNL

(iv) Differential effects on chronic minocycline treatment on genes encoding immune mediators in the PFC, amygdala and spinal cord following SNL in sham vs. OB
The effect of early life stress on affective and nociceptive behaviour prior to and following peripheral nerve injury in adulthood: examining accompanying alterations in neuroinflammatory mediators
6.1 Introduction

Adverse early life events are associated with a predisposition to developing psychiatric disorders (Gutman and Nemeroff, 2003, Nemeroff, 2004b) and chronic pain conditions (Fillingim and Edwards, 2005, Davis et al., 2005, Low and Schweinhardt, 2012) in later life. In order to determine if alterations in nociceptive responding observed in the olfactory bulbectomised rat model of depression were specific to this model, we examined nociceptive behaviour prior to and following peripheral nerve injury in an alternative model of affective disorders. Manipulation of mother-pup interactions has been used extensively as a model of early life stress in rodents with which to examine underlying neurobiological mechanisms (Cirulli et al., 2009). One of the most widely used methods involves the separation of the mother from her pups during the first 2 weeks of life, a critical period in the development of nociceptive, sensory, emotional and social functions (Fitzgerald, 2005, Lukas et al., 2010, Ellenbroek et al., 2005, Beggs et al., 2011). In rodents, two experimental procedures are primarily used: a prolonged single period (24hrs) of maternal deprivation (MD) (Ellenbroek et al., 2005, Viveros et al., 2009) and episodic brief periods (3–6hrs) of maternal separation (Lehmann and Feldon, 2000). A single episode of MD on postnatal day 9 results in behavioural changes in adolescence and adulthood which resemble those found in the affective disorders, including depressive-like responses (Llorente et al., 2007), enhanced impulsivity (Marco et al., 2007), disruption in prepulse inhibition (Ellenbroek et al., 2005, Ellenbroek and Cools, 2002) and cognitive deficits (Llorente et al., 2011, Marco et al., 2012). Despite the robust changes in affective behaviour, few studies have investigated the effect of early life stress on nociceptive responding. Episodic maternal separation has been shown to result in enhanced affective pain behaviour (Uhelski and Fuchs, 2010), visceral hypersensitivity (Coutinho et al., 2002, Chung et al., 2007b, Gosselin et al., 2010a, Moloney et al., 2012) and inflammatory hyperalgesia (Uhelski and Fuchs, 2010). However, the effect of a single period of MD on nociceptive responding has not been examined, nor have there been studies investigating the effect of early life stress on
neuropathic pain responding. The present study sought to address these two gaps in knowledge.

As highlighted in previous chapters, neuroinflammatory processes are now well recognised to play important roles in the pathophysiology of stress-related psychiatric disorders and chronic pain. Moreover, childhood stress is associated with increased peripheral inflammation in healthy individuals, depressed and schizophrenic patients (Danese et al., 2007, Miller et al., 2009b, Dennison et al., 2012, Pace et al., 2006). Stressful rearing conditions in rodents are associated with a number of neuroimmune alterations such as downregulated expression of microglial markers, cytokines (IL-10, IL-1β), chemokines (CCL7) and receptors (IL-5 receptor-alpha, CCR4) in the brain. In addition, early life stress reportedly reduces the expression of astrocytic markers (S100β, GFAP) in the anterior cingulate and precentral medial cortices (Musholt et al., 2009) and increases astrocyte density in the hippocampus and cerebellum (Llorente et al., 2009, Marco et al., 2012, Lopez-Gallardo et al., 2012). However, it is unknown if alterations in pro-inflammatory cytokines occur in key brain regions involved in emotional and nociceptive processing in the MD model. It is well established that neuropathic pain induces alterations in inflammatory processes at the level of the spinal cord (Ji et al., 2006, Schomberg and Olson, 2012). However, as discussed previously, peripheral nerve injury is also associated with increased supraspinal neuroinflammatory processes. Specifically, chronic constriction injury is associated with increased hippocampal TNFα levels (Ignatowski et al., 1999) while spared nerve injury is associated with increased IL-1β expression in the frontal cortex and GFAP expression in the periaqueductal grey (Norman et al., 2010b, Apkarian et al., 2006, del Rey et al., 2011). Moreover, we reported that SNL increased IL-10 in the thalamus and amygdala, and decreased IL-6 in the amygdala and hippocampus (Chapter 3). Chronic stress prior to peripheral nerve injury has been shown to exacerbate mechanical allodynia, depressive-like behaviour, and augment injury-induced IL-1β expression in the frontal cortex (Norman et al., 2010b) and nerve injury in OB rats was accompanied by increased cytokine and
glial mRNA expression in the PFC (Chapter 3&4), indicating a possible functional interaction between depression, neuroinflammation and pain. Thus, we hypothesised that early life stress-induced changes in affective and nociceptive behaviour may be accompanied by alterations in supraspinal neuroinflammatory processing. Childhood trauma has been associated with abnormalities in brain regions involved in emotion and pain, including an overall volume loss in the hippocampus and prefrontal cortex (Bremner et al., 1997, Stein et al., 1997, Carrion et al., 2001, Driessen et al., 2000). Moreover, the hippocampus and prefrontal cortex exhibit MD-induced alterations in a number of systems implicated in pain and affect, including neurotrophins, monoamines, glia, synaptic plasticity, and cannabinoids (Rentese et al., 2010, Roceri et al., 2002, Marco et al., 2012, Lopez-Gallardo et al., 2012, Llorente et al., 2010, Husum et al., 2002). Thus, we examined the effect of MD and SNL interactions on the expression of genes encoding inflammatory mediators in these two regions.

### 5.1.2 Hypothesis and aims

**Hypothesis**

Nociceptive and neuropathic pain-related responding would be enhanced following early life stress, in a sexually dimorphic manner, effects accompanied by altered affective behaviour and neuroimmune gene expression in the PFC and hippocampus

1. Examine the effect of MD on affective and nociceptive responding prior to and following peripheral nerve injury
2. Determine if MD-induced alterations in behavioural responding are associated with concomitant alterations at the neuroimmune level by evaluating the expression of genes encoding markers of glial activation and cytokines in the prefrontal cortex and hippocampus
3. Evaluate if MD elicits sexually dimorphic effects on affective behaviour, nociceptive responding and the expression of central inflammatory mediators following peripheral nerve injury
6.2 Materials and Methods

6.2.1 Animal husbandry

Experimental subjects were the offspring of Albino Wistar male and female rats purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain) mated (one male × two females) in the animal facility (Universidad Complutense Madrid) approximately 2 weeks after their arrival. Animals were housed in standard facilities on a reverse light-dark cycle (lights on at 20:00 h). On the day of birth (postnatal day [PND] 0), litters were sex-balanced, weighed and culled to 8 pups per dam (4 males and 4 females). Testing began in adulthood (>PND 69) and all testing was carried out during the dark phase.

The experimental protocol was carried out in accordance with the guidelines and approval of the Universidad Complutense Madrid institutional committee and in compliance with the Royal Decree 1201/2005, October 21, 2005 (BOE no. 252) on protection of experimental animals, and by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609.

6.2.2 Experimental design

The experimental design is presented in Fig. 6.1, with testing beginning in adulthood. Essentially, control and MD male and female animals (n=20/group) were tested in the holeboard test, elevated plus maze and open field test to assess exploration, anxiety-like behaviour and locomotor activity, respectively, while nociceptive responding was assessed using the hot plate test (noxious thermal stimulus, Section 6.2.6 of this chapter), von Frey test (mechanical stimulus, section 2.4.2.1) and the acetone drop test (cold stimulus, section 2.4.2.3). Animals were then allocated to one of the following groups: Control-non-spinal nerve ligation (Co-NSNL) (male n=8, female n=10), Control-spinal nerve ligation (Co-SNL) (male n=10, female
Chapter 6 – Early life stress and neuropathic pain

n=10), MD-NSNL (male n=10, female n=10), MD-SNL (male n=9, female n=9). Four animals were lost due to surgical complications (3 died post-surgery, and one had L4 damage and could not be tested for withdrawal responses and so was euthanized). Mechanical withdrawal thresholds were examined on days 1, 3, 7, 10 and 14 post-surgery. Responding in the acetone drop test was assessed on days 7 and 14 post-surgery. Animals were reassessed in the holeboard test (Section 6.2.4) and elevated plus maze (Section 6.2.5) on day 16, and in the open field test (Section 2.4.1.1) on day 18, post-surgery to examine if persistent pain had an effect on affective and exploratory behaviours. Animals were sacrificed by decapitation 48hrs following the final behavioural test (day 21 post-surgery). The prefrontal cortex and hippocampus were dissected out on an ice cold plate and stored at -80ºC until assessed for mRNA expression of inflammatory markers as described in Chapter 2 (Materials and Methods, Section 2.7).

Oestrous cycle was not controlled in the current study, due to concerns that the stressful nature of the procedure would impact on pain responding. Behavioural experiments were carried out in Madrid, Spain, while post-mortem studies were performed in Galway, Ireland. The maternal deprivation procedure had been characterised in Wistar rats, thus the strain used in this study is different to previous chapters (Sprague Dawley).

<table>
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<tr>
<th>MALES</th>
<th>FEMALES</th>
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<tr>
<td></td>
<td>NSNL</td>
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<tr>
<td>CONTROL</td>
<td>10</td>
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<td>MD</td>
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</tr>
</tbody>
</table>

Table. 6.1 Litters and group numbers

6.2.3 Maternal deprivation protocol

Maternal deprivation (MD) took place on PND9 as previously described (Llorente et al., 2007, Llorente et al., 2009, Ellenbroek et al., 1998). On PND9 (09.00h), the dam was removed from the home cage in the holding
room and placed in a novel clean cage with access to food and water. Meanwhile, pups were moved to a separate room (under white light conditions), body weight recorded, and the litter placed into a clean cage, with fresh bedding containing some sawdust from the home cage. The litter were returned to the holding room where they remained for the 24h deprivation period. The cages with the dam and its corresponding litter were placed beside each other on a separate rack from control groups. On PND 10 (09.00h), pups were weighed, and the dam and its litter were returned to the home cage. Control animals (Co) were housed in the holding room, and underwent the same procedure (weighing, clean cages) as MD animals, but were returned to the dam. Body weight was recorded and fresh bedding was provided from PND10 until PND64 every 6 days. Pregnant females and lactating dams had free access to water and to the specific breeding diet (A03; Panlab, Barcelona, Spain), and food pellets were changed to the adult diet at weaning (PND 22, A04; Panlab, Barcelona, Spain) when animals from the same litter were housed in pairs of siblings of the same sex.

### 6.2.4 Holeboard test

The holeboard test is used to measure exploratory and anxiety-like behaviour, with reduced duration of head dips interpreted as increased anxiogenic behaviour (Takeda et al., 1998). On the testing day, animals were habituated to a quiet room for a 30 minute period prior to experimental testing. The holeboard was a box (60 cm × 60 cm × 45 cm) with matte-painted metallic walls and a plastic-covered wooden floor bearing four equally spaced holes (3.8 cm in diameter) and divided into 36 squares (10 cm × 10 cm). Animals were placed in the centre of the holeboard arena and the duration of head-dipping, a measure of directed exploration (Ethovision XT, Noldus Netherlands) was assessed over a 5 min testing period. Testing occurred under red-light conditions (lux 0-1).

### 6.2.5 Elevated plus maze

Immediately following exposure to the holeboard test, animals were placed in the elevated plus maze which consisted of two open (50 cm × 10 cm) and
two enclosed arms with a central square area (10 cm × 10 cm), elevated to a height of 62 cm from the floor of the room. Frequency of entries and duration of time spent in the different arms was assessed manually over a 5 minute test period. A greater percentage of time and entries in the open arms of the elevated plus maze has been reported to be indicative of reduced anxiety-like behaviour, while the number of closed arms entries provides a measure of locomotor activity. Percentage open arm entries were calculated as number of open arm entries / total arm entries × 100.

6.2.6 Hot plate test
The hot plate test was used to assess nociceptive responding to a noxious thermal stimulus. On the test day, the animals were moved to the testing room to habituate for 30 minutes after which they were placed individually onto a hot plate (Harvard Apparatus) heated to 55 ± 1°C. Thermal nociception was measured as the time elapsed, i.e. latency to respond (seconds), between placement of the animal on the surface of the hot plate and when the animal first licked either of its hind-paws, or jumped. A cut-off time of 40s was employed in order to avoid any tissue damage.

6.2.7 Statistical analysis
PASW 18 statistical package was used to analyse all data. Kolmogorov and Levene tests were used to determine normality and homogeneity of variance, respectively, and data were analysed using two-way (factors of MD and sex or MD and SNL) or three-way (factors of MD, SNL and sex) analysis of variance (ANOVA) for parametric data, and Kruskal-Wallis for non-parametric data. Repeated measures ANOVAs were used to assess changes over time. Post-hoc analysis was performed using Duncan’s test for parametric data, and Mann-Whitney U-test for non-parametric data, where appropriate. Data were considered significant when P<0.05. Results are expressed as group means ± standard error of the mean (SEM).
Fig 6.1 Experimental Protocol. Abbreviations: AT Acetone-drop test; EPM Elevated Plus Maze, HB Holeboard, MD Maternal Deprivation, NSNL non-Spinal Nerve Ligation, PND Postnatal day, SNL Spinal Nerve Ligation, VF von Frey.
6.3 Results

6.3.1 MD induced long-lasting reductions in body weight in both male and female rats

Body weight (g) did not differ between the groups prior to MD on PND9. Two-way ANOVA revealed effects of MD ($F_{(1,75)} = 29.30 \ P < 0.001$) and sex ($F_{(1,75)} = 343.70 \ P < 0.001$) on body weight following MD (PND10), until the final assessment on PND64. Post-hoc analysis revealed that body weight of both male and female MD animals was significantly reduced following MD (Table 6.1). In addition, female rats (both control and MD) weighed significantly less than their male counterparts from PND28 onwards (Two-way ANOVA effect of Sex: $F_{(1,75)} = 343.70 \ P < 0.001$).

<table>
<thead>
<tr>
<th></th>
<th>Co-Male</th>
<th>MD-Male</th>
<th>Co-Female</th>
<th>MD-Female</th>
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<td>20.4±0.5</td>
<td>21.0±0.3</td>
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<td>82.4±1.4°</td>
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<td>PND48</td>
<td>210.9±2.9</td>
<td>193.1±3.0°</td>
<td>162.2±2.4°</td>
<td>151.0±2.2°</td>
</tr>
<tr>
<td>PND52</td>
<td>253.9±3.4</td>
<td>235.3±3.9°</td>
<td>181.6±2.6°</td>
<td>170.5±2.5°</td>
</tr>
<tr>
<td>PND58</td>
<td>291.3±4.7</td>
<td>268.6±5.4°</td>
<td>196.1±3.0°</td>
<td>183.5±3.1°</td>
</tr>
<tr>
<td>PND64</td>
<td>317.8±4.7</td>
<td>293.4±5.6°</td>
<td>206.9±2.9°</td>
<td>194.1±3.2°</td>
</tr>
</tbody>
</table>

Table 6.2. Body weight (g) of control and MD rats of both sexes, examined over the test period. °P<0.05 vs. corresponding Co. ‡P<0.05, §P<0.01 vs. corresponding male. Data are mean ± SEM (n = 20). Co Control, MD Maternal Deprivation, PND Postnatal day.
6.3.2 MD induces sex-specific changes in exploratory and anxiety-related behaviour

Two-way ANOVA revealed an effect of sex on head-dip duration in the holeboard ($F_{(1,71)} = 7.27, \ P=0.009$). Head-dip duration was increased in MD females when compared to MD males, indicating increased exploratory behaviour (Fig. 6.2A). Two-way ANOVA revealed an MD × sex interaction on percentage of open arm entries in the elevated plus maze ($F_{(1,62)} = 4.61, \ P = 0.035$, Fig. 6.2B). MD males exhibited increased percentage of open arm entries when compared to control males, indicative of reduced anxiety-like behaviour, an effect not observed in female rats. There were no significant differences between groups for the number of closed arm entries (Fig. 6.2C), indicating that the MD effects on anxiety-related behaviour in male rats were independent of effects on locomotor activity.

![Figure 6.2](https://example.com/fig62.png)

**Fig. 6.2 MD induces sex-specific changes in affective responding.** (A) Head-dip duration in the holeboard is increased in MD females when compared to MD males. (B) Percentage open arm entries in the elevated plus maze is increased in MD males when compared to control males. (C) Number of closed arm entries in the elevated plus maze is unaltered by MD or sex. $^#P<0.05$ vs. MD male, $^*P<0.05$ vs. Co male. Data expressed as Mean ± SEM, n=20.
6.3.3 Maternally deprived females show locomotor hyperactivity and anxiogenic behaviour in the open field

Two-way ANOVA revealed effects of MD \((F_{(1,75)} = 5.873, P=0.018)\) and sex \((F_{(1,75)} = 10.297, P=0.002)\) on distance moved in the open field. MD did not alter locomotor activity in males. In contrast, MD females showed increased distance moved when compared to both MD males and Co females (Fig 6.3A). There were effects of MD \((F_{(1,75)} = 6.08, P=0.016)\) and sex \((F_{(1,75)} = 8.874, P=0.004)\) on rearing duration in the open field with post-hoc analysis revealing that MD males exhibit increased rearing compared to Co counterparts (Fig 6.3B). In addition, Co females exhibited increased rearing compared to their male counterparts. There was no effect of MD or sex on number of faecal pellets produced (Fig 6.3C) or time spent in the inner zone of the open field (Fig 6.3D). However, there was an effect of MD on latency to enter the inner zone \((F_{(1,76)} = 5.528, P=0.021)\), with MD females exhibiting an increased latency to enter the inner zone, indicative of anxiogenic behaviour (Fig. 6.3E).
Fig. 6.3 Behaviour in the open field in control and MD males and females (A) Distance moved in the open field (B) Rearing duration (C) Faecal pellet count (D) Time in the inner zone (E) Latency to enter the inner zone. *P<0.05 vs. Co male, †P<0.05 vs. Co female, ‡P<0.05, §§P<0.01 vs. MD male. Data expressed as Mean ± SEM. N=20.
6.3.4 MD results in sexually dimorphic alterations in nociceptive responding in a stimulus-dependent manner

Examining the effect of MD on nociceptive responding revealed a significant MD × sex interaction ($F_{(1,76)} = 6.16$, $P = 0.015$) on latency to jump in the hot plate test with post-hoc analysis revealing that MD females, but not MD males, exhibit an increased latency to respond in comparison to control counterparts. Thus, MD females exhibit reduced sensitivity to a noxious thermal stimulus (Fig. 6.4A). In addition, control females exhibited reduced hot plate latencies (Fig 6.4A) when compared to control males, indicating increased sensitivity to a noxious thermal stimulus.

In the von Frey test, MD female, but not male, rats exhibited a significant reduction in mechanical withdrawal thresholds (MD $F_{(1,76)} = 13.31$, $P<0.001$; MD × sex interaction $F_{(1,76)} = 4.20$, $P=0.044$), when compared to control counterparts (Fig. 6.4B), indicative of mechanical allodynia. Kruskal-Wallis revealed an overall effect of group on the lowest filament to elicit a response in the von Frey test ($\chi^2_{(3)} = 18.31$, $P<0.001$). MD males and females responded at lower filaments when compared to controls, indicating mechanical allodynia (Fig 6.4C). Control females responded at lower von Frey filaments (Fig 6.4C) when compared to control males.

There was no effect of MD or sex on paw withdrawal latency or withdrawal frequency to an innocuous cold stimulus in the acetone drop test (Fig. 6.4D&E).
**Fig 6.4** Effect of MD and sex on nociceptive responding to heat, mechanical and cold (A) Latency to jump in the hot plate test (B) 50% Mechanical withdrawal thresholds of the hind-paws in the von Frey test. (C) Lowest filament to elicit a response in the von Frey test. (D) Paw withdrawal latency and (E) withdrawal frequency to respond to acetone application to the hind-paws. *P <0.05, **P<0.01 vs. Co, #P<0.05, ###P<0.01 vs. males. Data expressed as Mean ± SEM, n=20.
6.3.5 SNL-induced mechanical alldynia is enhanced in MD female, but not male, rats

Analysis of mechanical withdrawal thresholds revealed that SNL induces mechanical alldynia of the ipsilateral (left) hind-paw of both control and MD rats of both sexes when compared to NSNL counterparts (SNL: $F_{(1,65)} = 95.92$, $P < 0.001$, Fig. 6.5A-C). Further temporal analysis indicated a significant time $\times$ MD $\times$ sex $\times$ SNL interaction ($F_{(4,260)} = 2.45$, $P=0.047$) and post-hoc analysis revealed that SNL induced mechanical alldynia in both MD and control male and control female rats from day 1 post surgery, while mechanical alldynia was evident in MD females from day 3 post-surgery, when compared to pre-surgery values. MD did not alter mechanical withdrawal thresholds in SNL male rats at any of the time-points examined. However, on day 14 post-SNL, MD-SNL females exhibited lower withdrawal thresholds of the ipsilateral hind-paw compared to their control counterparts (MD-SNL vs. Co-SNL females). Analysis of the number of responses to the 1g von Frey filament at this time-point revealed that MD-SNL females exhibit an increased number of responses indicative of enhanced mechanical alldynia, when compared to both NSNL and control counterparts ($\chi^2_{(2)} = 14.76$, $P = 0.002$, Fig.6.5B inset). It should be noted that similar to the responses observed prior to surgery, both male and female MD animals which underwent sham surgery (NSNL) exhibited mechanical alldynia when compared to corresponding controls (MD: $F_{(1,69)} = 10.42$, $P = 0.002$; MD-NSNL vs. Co-NSNL Fig. 6.5C). In addition, control and MD-NSNL females demonstrated lower withdrawal thresholds compared to their male counterparts ($F_{(1,69)} = 19.53$, $P<0.001$, Fig. 6.5C).

SNL did not alter the mechanical withdrawal thresholds of the contralateral hind-paw of control or MD males. MD females displayed lower mechanical withdrawal thresholds of the contralateral hind-paw, an effect not altered by SNL (see appendix Fig. B.10).
Fig. 6.5 MD females exhibit exacerbated mechanical allodynia of the ipsilateral hind-paw post-SNL (A) 50% mechanical withdrawal thresholds of control and MD males. (Ai) Inset: % withdrawal response to 1g filament in males on Day 14 post surgery (B) 50% mechanical withdrawal thresholds of control and MD males. (Bi) Inset: % withdrawal response to 1g filament in males on Day 14 post surgery (C) Area under the curve of mechanical thresholds *P<0.05 **P<0.01 vs. NSNL, 'P<0.05 'P<0.01 vs. Co, †P<0.05 vs. males. Data expressed as Mean ± SEM, n=8-10.
6.3.6 SNL-induced cold allodynia is enhanced in MD females, but not males

Repeated measures three-way ANOVA revealed a significant effect of MD \( (F_{(1,68)} = 5.8, P=0.019) \), SNL \( (F_{(1,68)} = 109.95, P<0.001) \), MD × sex × SNL interaction \( (F_{(1,68)} = 4.55, P=0.037) \), time \( (F_{(2,136)} = 88.52, P<0.001) \), time × SNL interaction \( (F_{(2,136)} = 16.26, P<0.001) \) on latency to respond to acetone application to the ipsilateral hind-paw. Cold allodynia, expressed as a decrease in the paw withdrawal latency was observed in the ipsilateral hind-paw of both control and MD rats, 7 and 14 days following SNL, when compared to NSNL controls (Fig 6.6A&C). For change in withdrawal frequency from prior to and following SNL, Friedman’s test revealed a significant overall effect of time \( (\chi^2_{(2)} = 69.71, P<0.001) \). Post-hoc tests revealed a significant effect of group on day 7 \( (\chi^2_{(7)} = 52.57, P<0.001) \) and day 14 \( (\chi^2_{(7)} = 55.58, P<0.001) \) following NSNL/SNL surgery. MD-SNL female rats exhibited increased withdrawal frequency on day 14 when compared to control counterparts, indicating an MD-induced exacerbation of cold allodynia in female rats. When compared to pre-SNL values, control female, MD male and female NSNL animals exhibited a reduced response latency (but not frequency) of the ipsilateral hind-paw, an effect not seen in control NSNL males. However, this effect was much less pronounced than that observed in SNL groups (Fig 6.6A&C).

MD-SNL males exhibited reduced paw withdrawal latency and increased withdrawal frequency to acetone application to the contralateral hind-paw on day 7 post-SNL when compared to Co-SNL males (see appendix Fig. B.11), indicating contralateral cold allodynia. MD-SNL females exhibited reduced paw withdrawal latency and increased withdrawal frequency to acetone application to the contralateral hind-paw on day 14 post-SNL when compared to Co-SNL females, indicating contralateral cold allodynia (see appendix B.11). This effect was much less pronounced than that observed in the ipsilateral hind-paw.
Fig. 6.6 MD females exhibit exacerbated cold allodynia of the ipsilateral hind-paw following SNL (A) Paw withdrawal latency and (B) withdrawal frequency to acetone application in males (C) Paw withdrawal latency and (D) withdrawal frequency to acetone application in females. *P<0.05, **P<0.01 vs. NSNL, +P<0.05, ++P<0.01 vs. Co. Data expressed as Mean ± SEM, n=8-10.
6.3.7 Effect of MD-SNL interactions on exploratory and anxiety-like behaviour

Three-way ANOVA revealed effects of MD ($F_{(1,67)} = 6.75$, $P = 0.012$) and sex ($F_{(1,67)} = 15.49$, $P < 0.001$) on duration of head dipping in the holeboard test following SNL. Re-exposure to the holeboard revealed increased duration of head dipping in MD female, but not male, rats (Fig. 6.7A), an effect not altered by SNL. In the elevated plus maze, three-way ANOVA revealed an effect of sex ($F_{(1,62)} = 7.13$, $P = 0.01$). MD-SNL males exhibited decreased percentage open arm entries compared to MD-SNL female counterparts (Fig. 6.7B). There were no significant differences between groups for the number of closed arm entries (data not shown). Three-way ANOVA revealed effects of MD ($F_{(1,62)} = 8.214$, $p = 0.006$), sex ($F_{(1,62)} = 14.3$, $P < 0.001$) and SNL ($F_{(1,62)} = 5.597$, $P = 0.021$) on distance moved in the open field following NSNL/SNL surgery. MD resulted in increased locomotor activity in males, an effect not altered by SNL (6.7C). In addition, control females exhibited increased locomotor activity, an effect not altered by SNL. Finally, SNL in MD females resulted in reduced locomotor activity compared to their MD-NSNL counterparts (Fig. 6.7C). An effect of sex on rearing duration ($F_{(1,64)} = 34.961$, $P < 0.001$) was revealed with post-hoc analysis confirming that females exhibit a greater amount of time rearing when compared to males, an effect not significantly altered by MD or SNL (see appendix Fig. B.12). There were no effects of MD, sex or SNL on time in the inner zone, latency to enter the inner zone or pellet count (data not shown).
Fig. 6.7 Behaviour in the holeboard, elevated plus maze and open field following NSNL/SNL surgery. (A) Head dip duration in the holeboard (B) Percentage of open arm entries in the elevated plus maze (C) Distance moved in the open field. #P<0.05 effect of sex, +P<0.05 effect of MD, *P<0.05 effect of SNL. Data expressed as Mean ± SEM, n=8-10.
6.3.8 MD results in reduced mRNA expression of proinflammatory cytokines in the prefrontal cortex following SNL in a sex-dependent manner

Analysis of the expression of glial activation markers and pro-inflammatory cytokines in the PFC revealed that control females exhibit decreased expression of GFAP (Sex: $F_{(1,66)} = 5.55 \ P = 0.022$) and IL-1β (Sex: $F_{(1,67)} = 19.20 \ P < 0.001$) mRNA when compared to male counterparts (Co-NSNL female vs. Co-NSNL male; Fig. 6.8 B-C). MD per se did not alter the mRNA expression of any of the inflammatory markers in either male or female rats (MD-NSNL vs. Co-NSNL). However, IL-6 mRNA was reduced in MD-SNL males when compared to control-SNL counterparts (MD: $F_{(1,64)} = 8.26, \ P = 0.005$; MD-SNL male vs. Co-SNL male; Fig 6.8E). In addition, TNFα mRNA was reduced in MD-SNL females when compared to male counterparts (MD × sex $F_{(1,65)} = 4.13, \ P = 0.046$; MD-SNL females vs. MD-SNL males; Fig 6.8D), an effect not observed in NSNL animals. Sex, MD or SNL did not alter CD11b mRNA expression.
Fig. 6.8 The expression of (A) CD11b, (B) GFAP, (C) IL-1β (D) IL-6 and (E) TNFα mRNA in the prefrontal cortex. *P<0.05 vs. males, **P<0.05 vs. control. Data expressed as Mean ± SEM, n=8-10.
6.3.9 MD-SNL exhibit increased mRNA expression of neuroimmune markers in the hippocampus in a sex-dependent manner

Although neither MD nor SNL alone altered the expression of any of the inflammatory mediators under investigation, MD-SNL male rats exhibited increased expression of GFAP ($F_{(1,64)} = 4.9$, $P=0.023$) and IL-1β (SNL: $F_{(1,62)} = 5.55$, $P=0.041$; SNL × sex: $F_{(1,62)} = 7.77$, $P=0.007$) mRNA in the hippocampus, compared with NSNL counterparts (MD-SNL male vs. MD-NSNL male; Fig 6.9 B-C). In comparison, MD-SNL female rats displayed increased expression of IL-6 mRNA when compared to NSNL counterparts (MD × SNL × sex: $F_{(1,61)} = 4.56$, $P=0.037$, MD-SNL vs. MD-NSNL; Fig 6.9E) and increased TNFα mRNA expression when compared to control counterparts (MD: $F_{(1,62)} = 4.10$, $P=0.048$; MD × SNL: $F_{(1,61)} = 5.4$, $P=0.023$; MD-SNL vs. Co-SNL; Fig 6.9D).
Fig. 6.9 The expression of (A) CD11b, (B) GFAP, (C) IL-1β (D) IL-6 and (E) TNFα mRNA in the hippocampus. *P<0.05 vs. NSNL, †P<0.05 vs. control. Data expressed as Mean ± SEM, n=8-10.
6.4 Discussion

The present study demonstrates that early life stress, in the form of maternal deprivation (MD), results in sex-dependent alterations in affective behaviour and nociceptive responding to mechanical and thermal stimuli prior to and following peripheral nerve injury in adulthood. In particular, MD female rats exhibit thermal (heat) hypoalgesia and robust mechanical allodynia at baseline, and display enhanced mechanical and cold allodynia following SNL. Furthermore, the expression of pro-inflammatory cytokines and astrocytes in key brain regions involved in affective and nociceptive processing, the PFC and hippocampus, is differentially altered in male and female rats exposed to a combination of MD and SNL. These data provide evidence for sexually dimorphic effects of early life stress on nociceptive processing and suggest that alterations in neuroinflammatory processes may, in part, underlie these effects.

6.4.1 Long-term effects of maternal deprivation on body weight, locomotor activity and affective behaviour

As previously reported (Viveros et al., 2010, Viveros et al., 2009, Llorente-Berzal et al., 2011, Husum et al., 2002, Yamazaki et al., 2005), MD induced long-lasting reductions in body weight compared to controls. Changes in developing hypothalamic circuits involved in the control of energy balance have been proposed to underlie these alterations in body weight (Viveros et al., 2010, Llorente-Berzal et al., 2012, Llorente et al., 2010), and variations in maternal care in the post-deprivation period may also be involved (Ellenbroek and Cools, 2002). A number of studies have reported sex-dependent alterations in locomotor activity and affective behaviour following early life stress. The present data demonstrated that MD females exhibit increased locomotor activity in the open field when compared with male counterparts, an effect reported in some (Takase et al., 2012) but not all (Llorente-Berzal et al., 2011, Llorente-Berzal et al., 2012) studies. It is possible that the stress associated with exposure to a novel arena under a high level of illumination such as in the current study (180 lux) and in that
reported by Takase et al. (150 lux), may have uncovered the hyperactivity response in MD females. In addition, MD females exhibited increased latency to enter the inner zone of the open field, indicating possible anxiogenic-like behaviour. However, in the EPM, MD females did not exhibit anxiogenic-like behaviour, an effect which may again due to the low vs. high level of illumination. The lack of novelty and repeated behavioural testing may account for the absence of anxiety-like behaviour in females after re-exposure to the open field following NSNL surgery. In comparison, MD males exhibited increased rearing prior to surgery, but exhibited increased distance moved in the open field on re-exposure. Moreover, sex-specific effects were observed in the holeboard test with MD females exhibiting enhanced directed exploration, as demonstrated by increased head-dip duration, when compared to MD males. This finding correlates with previous reports (Llorente-Berzal et al., 2011) and remains following SNL. These data indicate that differential effects of MD on locomotor activity and affective behaviour exhibited in adulthood may depend on the sex of the animal, and the stress and novelty of the testing paradigm.

6.4.2 Sex-dependent alterations in nociceptive responding to mechanical and thermal stimuli in maternally deprived rats

Assessing the effects of sex on nociceptive responding revealed that female (control) rats exhibit lower response thresholds to heat and mechanical stimulation (but not cold) when compared to males, supporting evidence that females exhibit greater sensitivity to experimental pain than males (Fillingim et al., 2009). Our data also demonstrate that early life stress differentially alters nociceptive responding depending on stimulus modality and the sex of the animal. Although several studies have demonstrated the effect of early life stress on visceral pain (Moloney et al., 2012, Chung et al., 2007b, Coutinho et al., 2002), the present study is one of the few studies that have examined its effect on thermal or mechanical nociceptive responding. Maternal separation has been demonstrated to increase the
thermal response latency of female (Weaver et al., 2007), but not male (Uhelski and Fuchs, 2010), rats in the hot plate test. In accordance with these findings, the present study demonstrated that MD female, but not male, rats exhibit increased nociceptive thresholds to a noxious heat (thermal hypoalgesia), but not cold, stimulus. In addition, MD males and females displayed mechanical allodynia, an effect more robustly displayed in females. To date, the effect of early life stress on mechanical thresholds has only been reported in male animals. Exposure to the neonatal limited bedding paradigm results in lower mechanical nociceptive thresholds of the muscle (but not skin) in male rats during adulthood (Green et al., 2011, Alvarez et al., 2013). In comparison, mechanical withdrawal thresholds were unaltered in maternally separated adult males (Uhelski and Fuchs, 2010). To our knowledge, the present study is the first to demonstrate sexually-dimorphic effects of MD on nociceptive responding to thermal and mechanical stimuli.

6.4.3 Maternally deprived females, but not males, exhibited enhanced SNL-induced mechanical and cold allodynia

Due to the link between early life stress and chronic pain, a further aim of this study was to determine the effect of maternal deprivation on the development and maintenance of a chronic, persistent, clinically relevant pain state, using the L5-L6 SNL neuropathic pain model. In agreement with previous reports and similar to that reported in previous chapters (3-5), SNL induced robust mechanical and cold allodynia, an effect observed in both control and MD animals. However, MD females were slower to develop mechanical allodynia following SNL when compared to controls (day 3 vs. day 1). Moreover, MD females also exhibited enhanced mechanical and bilateral cold allodynia of the ipsilateral hind-paw 14 days following SNL compared with control females. In Chapter 3 and 5, OB male rats exhibited enhanced cold, but not mechanical allodynia following SNL and previous studies have reported enhanced mechanical allodynia to peripheral nerve injury following chronic restraint stress and in the Wistar-Kyoto rat model of depression (Zeng et al., 2008a, Norman et al., 2010b). However, to our
knowledge, this is the first study to examine the effect of early life stress on neuropathic pain behaviour in rodents, demonstrating sex-dependent effects of MD on nociceptive responding following peripheral nerve injury.

6.4.4 Effect of MD and SNL on locomotor activity and affective behaviour

Alterations in affective responding such as anxiety- and depressive-like behaviours have been shown to be induced in a number of models of peripheral nerve injury (Yalcin et al., 2011, Suzuki et al., 2007, Norman et al., 2010b, Narita et al., 2006a), although in the present study SNL failed to modulate affective behaviour. The short period following surgery over which these behaviours were examined (Day 16), may account for the lack of effects as previous studies have indicated that alterations in affective behaviour only manifest after at least a month post-surgery (Yalcin et al., 2011, Suzuki et al., 2007, Narita et al., 2006a). Moreover, differences in strain (SD vs. Wistar), housing (individual vs. paired), and light-dark cycle between the current study and Chapter 3 may account for the lack of SNL-induced anxiety-related behaviour seen here. In contrast, MD-SNL males exhibited reduced time in the open arms of the EPM when compared to their female counterparts, which indicates that SNL induces anxiety-like behaviour in males exposed to early life stress. Moreover, SNL resulted in reduced locomotor activity in the open field of MD-SNL females. Thus, SNL induces sex-dependent alterations in affective behaviour and locomotor activity in MD under conditions that control animals are unaffected.

Although the neurobiological mechanisms underlying the effects of MD on nociceptive responding in adulthood remain to be determined, MD induces a range of sex-dependent developmental and psychoneuro-immunoendocrine alterations (Viveros et al., 2009). It has been proposed that disruptions in stress regulation caused by early life stress may explain the reduced pain thresholds seen in fibromyalgia (Low and Schweinhardt,
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Noxious painful stimuli in early life have been demonstrated to increase the plasticity of nociceptive systems, altering the development of somatosensory pathways and subsequently leading to long-lasting alterations in nociceptive responding to thermal and inflammatory stimuli in adulthood, with females displaying greater responses compared to males (LaPrairie and Murphy, 2010, Fitzgerald, 2005). Thus, stressful events at critical developmental periods may cross-sensitise nociceptive circuits leading to enhanced susceptibility to chronic pain in later life. Therefore, it is possible that MD at a critical developmental stage (PND9) induces alterations in sensory and affective processing resulting in enhanced nociceptive responding in later life. The exacerbation of SNL-induced mechanical and cold allodynia in MD females suggests that adverse early life events may have a more profound impact on females, accounting for the increased incidence and severity of chronic pain conditions reported in this gender (Fillingim et al., 2009).

6.4.5 **MD and SNL interactions differentially alter astrocyte and cytokine expression in the PFC and hippocampus in a sex-dependent manner**

The PFC and hippocampus are key regions in both emotion and pain processing (Lorenz et al., 2003, del Rey et al., 2012) and several lines of evidence indicate that sex-dependent changes in neuronal and non-neuronal functioning in these regions may underlie the higher incidence of certain psychiatric and pain disorders in women (Fillingim et al., 2009, Parker and Brotchie, 2010). The present study demonstrated an effect of sex on the expression of inflammatory mediators in the PFC, specifically that female rats (control-NSNL) exhibit decreased GFAP and IL-1β mRNA expression in the PFC, but not hippocampus, when compared to male counterparts. Similarly, recent studies have demonstrated reduced GFAP density in adolescent female rats in the frontal cortex, but not hippocampus (Marco et al., 2012). Astrocyte number and density have been reported to be reduced in the amygdala (Johnson et al., 2012) and cerebellum (Suarez et al., 1992) of female animals when compared to males. Although further studies are required to determine the functional significance of such sex-dependent
alterations, it is possible that reduced astrocyte activity may underlie the lower withdrawal thresholds to mechanical stimuli in female vs. males rats observed in the current study. Astrocytes play an active role in the maintenance and modulation of synaptic transmission and plasticity, processes modulated by oestrogen (Garcia-Segura et al., 1999), which may impact upon prefrontal regulation of descending control of pain.

In the present study, MD per se did not alter GFAP or CD11b mRNA expression, markers of astrocyte and microglial activation respectively, in the hippocampus or PFC of adult rats. Previous reports have described increased astrocyte density in the hippocampus and cerebellum, with a slight increase in the PFC, of neonatal MD male rats (Llorente et al., 2009, Marco et al., 2012, Lopez-Gallardo et al., 2012). Different methods of evaluation of GFAP (qRT-PCR vs. immunohistochemistry) and time of evaluation (neonate vs. adult) may account for the discrepancies between the current study and previous reports.

Nerve injury has been shown to increase GFAP and cytokine expression (IL-1β) in the PFC, hippocampus and brainstem (Apkarian et al., 2006, Norman et al., 2010b, del Rey et al., 2012). In Chapter 3, we demonstrated that SNL decreases IL-6 expression in the amygdala but does not alter expression of pro-inflammatory cytokines in other brain regions, including the PFC and hippocampus. Similarly, SNL per se did not alter the expression of any of the inflammatory mediators under investigation in the PFC or hippocampus in the present study. It is possible that the post-surgery timepoint at which brain tissue was collected was not optimal to detect SNL-induced alterations in these pro-inflammatory mediators. However, the combination of peripheral nerve injury and MD resulted in sexually dimorphic alterations: reduced IL-6 expression in the PFC and increased GFAP and IL-1β expression in the hippocampus of MD males following SNL. In comparison, MD-SNL female rats exhibited lower TNFα in the PFC and enhanced IL-6 and TNFα expression in the hippocampus. To our
knowledge, this is the first study to examine and demonstrate the effect of early life stress on behavioural and supraspinal neuroimmune changes following peripheral nerve injury. The differential effect of MD-SNL on cytokine expression in male versus female brain regions involved in regulating emotion and pain may underlie the sexually dimorphic effects observed on nociceptive processing following SNL, effects which remain to be determined. Although it was beyond the scope of the current dissertation, future studies could examine if hormone levels (oestradiol, progesterone) are altered control vs. MD animals, effects which may explain the sexually dimorphic effects seen herein, as sex steroids have been shown to modulate central cytokine levels (Sarkaki et al., 2013).

Although MD per se did not alter neuroimmune mediators in the brain regions investigated, it is possible that MD may prime the neuroimmune system such that subsequent stressors such as peripheral nerve injury may elicit an exaggerated response. Glucocorticoids have been shown to mediate microglial priming (Frank et al., 2011) and short-term increases in glucocorticoid activity have been reported in MD animals (Llorente et al., 2008). Furthermore, MD females demonstrate increased susceptibility to the inflammatory autoimmune disease, experimental autoimmune encephalomyelitis (Teunis et al., 2002), lending further support for early-life stress-induced glial priming. Thus, sexually dimorphic dysregulation of glial-neuronal interactions may account for the heightened mechanical and cold allodynia observed in female rats following SNL.

6.5 Conclusion

In conclusion, MD induces sexually dimorphic effects on nociceptive and affective behaviour. Specifically, MD results in heightened mechanical and cold allodynia following peripheral nerve injury in female rats, effects accompanied by increased cytokine (IL-6 and TNFα) expression in the hippocampus. These findings provide further evidence for altered nociceptive responding prior to and following peripheral nerve injury in a
model of affective disorders, and suggest that neuroimmune alterations in brain regions responsible for processing emotion and pain may be implicated.

6.6 Summary of aims and subsequent findings

(1) Examined the effect of MD on affective and nociceptive responding prior to and following peripheral nerve injury

(i) Sex-dependent effects of MD on affective and nociceptive responding prior to and following peripheral nerve injury

(2) Determined if MD-induced alterations in behavioural responding are associated with concomitant alterations at the neuroimmune level by evaluating the expression of genes encoding markers of glial activation and cytokines in the prefrontal cortex and hippocampus

(ii) Sex-dependent alterations in neuroimmune gene expression in the PFC and hippocampus following the combination of MD and SNL.
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General discussion
The relationship between depression and pain and the neurobiological mechanisms underlying this association are far-removed from being completely comprehended. Understanding of the interaction between pain and depression may provide new knowledge on both or either conditions. It may be inferred that depression and chronic pain are distinct but related manifestations of underlying pathophysiological events driven by shared pathways. Supporting the antecedent hypothesis (that depression precedes the development of pain), a number of studies have shown that the presence of a depressive disorder significantly increases the risk of developing chronic neck, back, musculoskeletal, chest pain and headache (Carroll et al., 2004, Hotopf et al., 1998, Croft et al., 1995, Leino and Magni, 1993, Larson et al., 2004, Magni et al., 1994, Currie and Wang, 2005, Von Korff et al., 1993). The development of a chronic pain condition in an individual who suffers depression is likely to have a significantly negative impact on suffering in both domains. Over the past few years, an increasing body of research has provided some insight into the potential mechanisms that may mediate the link between the development of anxiety- and depressive-like behaviour in chronic pain conditions, however considerably less research has examined the impact of depression on the development of chronic pain.

In this dissertation, the aim was to examine nociceptive and neuropathic-pain behaviour in animal models of altered affect (the OB model of depression and maternal deprivation) and to examine if behavioural changes were accompanied by alterations at the monoaminergic and/or neuroimmune level in limbic and cortical regions responsible for the modulation of emotion and pain. This discussion focuses on appraising the most significant behavioural, pharmacological and neurobiological findings and how these contribute to increasing our understanding of the neurobiology underpinning depression, pain and their interaction.

The major contributions of this thesis include: (1) Characterisation of nociceptive and neuropathic pain responding to mechanical and thermal stimuli in two models of affective disorders; (2) Identifying neuroimmune alterations in discrete brain regions responsible for the processing of pain.
and emotion; (3) demonstrating differential behavioural responding and neuroimmune mRNA expression in response to chronic antidepressant and anti-inflammatory treatment in the presence of a depressive-like phenotype.

7.1 Altered nociceptive behaviour in the olfactory bulbectomised and maternal deprivation models prior to and following peripheral nerve injury

In Chapter 3, 4 and 5 (study 1 and 2), we confirmed previous findings from our laboratory (Burke et al., 2010) and others (Rodriguez-Gaztelumendi et al., 2006) that OB rats are more sensitive to mechanical stimuli applied to the hind-paw. These data suggest that mechanical allodynia is an important component of the OB syndrome. This provides a robust and reproducible behavioural output in which to examine the mechanisms underlying allodynia associated with a depressive-like phenotype and to develop new treatments that target depression associated with mechanical hypersensitivity. In addition, this work extends the nociceptive profile exhibited by OB rats by providing evidence for cold allodynia (Chapter 3 & 5) and thermal hyperalgesia (Chapter 5) in the model, however these effects are not consistently observed and as such may not be as robust as that of mechanical allodynia. As discussed (Section 1.3), other models of depression which also exhibit mechanical, cold and heat hypersensitivity include chronic mild stress (Bravo et al., 2012), chronic restraint stress (Spezia Adachi et al., 2012, Bardin et al., 2009), repeated reserpine (Arora et al., 2011, Nagakura et al., 2009) and chronic social defeat/stress (Rivat et al., 2010, Kim et al., 2012). Thus, enhanced nociceptive processing appears to be a hallmark of several models of depression, the magnitude and response of which depends on the model in question and stimulus applied. Expanding on these findings, this study also demonstrated for the first time that somatic nociceptive behaviour was altered in the MD model of early life stress in a stimulus- and sex-dependent manner. In Chapter 6, we observed thermal hypoalgesia in MD females, mechanical allodynia in MD
animals of both sexes (more robust in females) and no change in responding to a cold innocuous stimulus. These data are in accordance with the literature showing that early life stress in the form of repeated maternal separation (Weaver et al., 2007, Stephan et al., 2002, Coutinho et al., 2002) and neonatal isolation (Imanaka et al., 2008) results in thermal hypoalgesia; and that the neonatal limited bedding model results in increased responding to mechanical stimuli (Green et al., 2011, Alvarez et al., 2013). Thus, the effects of early life stress depend on the stimulus and type/duration of stressor. The current work expands on these data by indicating clear sexual dimorphisms in behavioural responding, which should be taken into consideration for future research. The studies presented in this thesis are in accordance with clinical reports that have shown that depressed patients exhibit increased sensitivity to mechanical, heat and cold stimuli applied to the skin (Strigo et al., 2008b, Borckardt et al., 2005, Paul-Savoie et al., 2011, Chiu et al., 2005, Hennings et al., 2012, Euteneuer et al., 2010, Gormsen et al., 2004, Normand et al., 2010) and that early life stress is associated with thermal hypoalgesia in women (Fillingim and Edwards, 2005). Thus, the two models (OB and MD) may be used to examine the underlying neurobiological mechanisms underpinning altered nociceptive responding in depression and following early life stress in greater detail.

The work presented herein describes the first characterisation of neuropathic pain behaviour in the OB rat. The study presented in Chapter 6 is also the first to examine neuropathic pain responding following early life stress. These data indicate exacerbated neuropathic pain-like behaviour in two models of affective disorders. Specifically, SNL-induced cold allodynia was heightened in the OB model (Chapter 3 & 5) and in MD female, but not male, rats (Chapter 6). Moreover, SNL-induced mechanical allodynia was exacerbated in MD females, an effect not seen in males, further highlighting the need to examine sexual dimorphisms in disease models. These data are supported by recent studies showing that the Wistar-Kyoto genetic model of depression exhibited exacerbated mechanical allodynia following CCI (Zeng et al., 2008a) and that prior exposure to chronic restraint stress
Chapter 7 – General Discussion

exacerbated spared nerve injury-induced mechanical allodynia (Norman et al., 2010b). In comparison, although CCI-induced mechanical and cold allodynia was not altered following social isolation and chronic unpredictable stress, the affective component of pain as measured in the place escape/avoidance paradigm was significantly enhanced in these models (Bravo et al., 2012, Bravo et al., 2013). In contrast, the Flinders sensitive line and UCMS models of depression showed decreased sensitivity to mechanical and thermal stimuli following SNL and partial sciatic nerve ligation, respectively (Shi et al., 2010a, Shir et al., 2001). Thus, different models of depression exhibit increased, decreased and no change in sensitivity to mechanical and thermal stimuli following peripheral nerve injury. However, the aforementioned studies all used male rodents, therefore it would be of interest to examine if neuropathic pain responding is altered in females in these models. The studies presented in this thesis demonstrate that nociceptive responding following spinal nerve ligation is altered in a modality-specific and sex-dependent manner in rodent models of depression and early life stress, thereby expanding our current understanding on affective modulation of pain.

7.2 Neuroimmune alterations in discrete brain regions responsible for the processing of pain and emotion in the OB and MD models

In Chapter 3, we reported a number of interactions between OB and SNL in neuroimmune mediators. First, we described a bilateral increase in GFAP in the spinal cord of OB animals, an effect which correlated significantly with mechanical withdrawal thresholds suggesting that increased SNL-induced neuroimmune activation in the spinal cord of OB rats may, at least in part, underlie the bilateral mechanical allodynia exhibited in this study. Increasing evidence indicates that supraspinal glial alterations play essential roles in depression and chronic pain. Some of the most interesting data from these experiments was that OB rats exhibited a reduced latency and number of responses to an innocuous cold stimulus following SNL, an effect
positively correlated with IL-6 and IL-10 mRNA expression in the amygdala. Therefore, altered nociceptive responding to a cold stimulus following SNL in OB animals is associated with, and possibly mediated by altered cytokines in the amygdala. Moreover, the combination of OB and SNL resulted in heightened expression of markers of microglia and astrocytes and pro- and anti-inflammatory cytokines in the PFC, changes which were replicated in Chapter 4 (CD11b, GFAP, IL-10), and in Chapter 5 (GFAP, similar trend for IL-10), indicating that neuroinflammation is a hallmark of OB-SNL and may in part underlie the behavioural changes observed. Any discrepancies on the particular cytokine changes observed between studies are most likely related to the slightly different experimental timelines and design between studies. Nonetheless, these data suggest that increased GFAP (and secondly IL-10) appears to be the most enduring and reproducible change observed in the PFC following the combination of OB and SNL. These data are interesting given that spinal astrocytes have been implicated in the maintenance of neuropathic pain (Raghavendra et al., 2003), and supraspinal astrocytes are implicated in descending pain facilitation (Wei et al., 2008). However, it must be noted that GFAP was not increased in the PFC of sham-SNL animals despite displaying mechanical and cold allodynia. It is possible that SNL induced an increase in these cytokines in sham animals at earlier time points or in different brain regions than examined in this study. Furthermore, we observed that following MD and SNL, expression of neuroinflammatory mediators is altered in the PFC and hippocampus in a sex-dependent manner. MD-SNL males exhibited increased GFAP and IL-1β mRNA expression in the hippocampus and decreased IL-6 expression in the PFC. In comparison, MD-SNL females exhibited increased IL-6 and TNFα in the hippocampus with a reduction in TNFα expression in the PFC. Although the direction of changes in the PFC is opposite to that seen in OB-SNL animals, the combination of MD and SNL resulted in differential astrocyte and cytokine expression depending on the brain region which is in accordance with the OB-SNL model, and extend this by providing sex-dependent alterations. Thus, the two models used in this thesis both demonstrate altered neuropathic pain behaviour
which is accompanied by differential immune mediator expression in
discrete brain regions responsible for processing emotion and pain.
Although the functional significance of altered immune gene expression in
these models remains to be determined, these data expand on other reports
that have shown enhanced inflammatory mediator mRNA and protein in the
brain of rodent models exhibiting depressive-like behaviour and persistent
pain. That is, depressive-like behaviour and spared nerve injury is
associated with enhanced IL-1β mRNA expression in the frontal cortex and
increased GFAP mRNA in the PAG (Norman et al., 2010b), socially
isolated mice with nerve injury and depressive-like behaviour exhibited
increased IL-1β mRNA in the frontal cortex (Norman et al., 2010a), nerve
injury in Wistar-Kyoto rats resulted in increased IL-1β mRNA in the
brainstem and PFC (Apkarian et al., 2006), reserpine-induced pain-
depression syndrome is associated with increased TNFα and IL-1β protein
in the cortex and hippocampus (Arora et al., 2011, Xu et al., 2013), and
inflammatory pain induced-depressive behaviour is associated with
increased IL-6 mRNA in the hippocampus (Kim et al., 2012). As such, the
current data show that the combination of depression and pain results in
increased neuroinflammation in discrete brain regions responsible for the
processing of emotion and nociception.

7.3 Examining monoamines in discrete brain regions responsible for the
processing of pain and emotion in the OB rat
Alterations in monoamines are proposed to regulate affect and pain,
however, in Chapters 3 and 4 we observed minimal alterations in the levels
of noradrenaline, serotonin and dopamine and their metabolites in the brain,
an effect that may be due to the time point at which these were examined.
One interesting finding was that the combination of OB and SNL resulted in
reduced concentration of 5-HT and 5HIAA in the hippocampus (Chapter 3),
suggesting that impaired serotonergic neurotransmission in the
hippocampus may be implicated in altered nociceptive responding in the OB
model. Modulation of the monoaminergic system with amitriptyline (a serotonin and noradrenaline reuptake inhibitor) further explored the involvement of this system in depressive-like and nociceptive responding, which will be discussed in detail below.

7.4 Differential behavioural responding in response to chronic antidepressant and anti-inflammatory treatment in the presence of a depressive-like phenotype

Chronic treatment with amitriptyline (a tricyclic antidepressant and first-line treatment for neuropathic pain) and minocycline (an inhibitor of microglial activation) elicited an antidepressant-like effect but did not alter basal nociceptive responding in the OB rat (Chapter 4 & 5). Moreover, these treatments are without effect on either affective or nociceptive responding in the absence of a depressive-like phenotype. It is possible that nociceptive responding is less responsive to this type of treatment and a longer duration may be required to normalise mechanical allodynia in OB rats. In addition, a clinical study has shown that chronic duloxetine (selective serotonin and noradrenaline reuptake inhibitor) treatment normalised thermal hypoalgesia but not ischaemic hyperalgesia in depressed patients despite concurrently reducing depressive symptoms in these patients (Bar et al., 2011), thus effects of antidepressants on nociceptive responding in depression may be stimulus-dependent. These data indicate that nociception and depression may be mediated by separate mechanisms, but show that two pharmacological treatments targeting primarily the monoaminergic system and microglial activation successfully attenuated OB-induced hyperactivity.

Chronic amitriptyline (AMI) and minocycline treatment differentially altered neuropathic pain-related behaviour in the presence or absence of a depressive-like phenotype. An important observation of our data was that chronic AMI attenuated neuropathic pain behaviour in a modality-specific manner in sham vs. OB animals. Specifically, AMI attenuated SNL-induced cold allodynia and thermal hyperalgesia but not mechanical allodynia in
sham animals, effects correlating with those previously reported in the SNL model (Pradhan et al., 2010, Esser et al., 2001). In direct contrast, AMI attenuated SNL-induced mechanical allodynia but not cold allodynia or thermal hyperalgesia in OB rats. These data highlight that modulation of monoaminergic neurotransmission elicits stimulus-specific effects on neuropathic pain responding depending on the presence or absence of a depressive phenotype. While the precise mechanism underlying this effect is unknown, SNL is associated with decreased 5-HTT and increased noradrenaline transporter density in the spinal cord, and increased 5-HTT density in forebrain and midbrain regions (Rojo et al., 2012). In addition, depletion of spinal 5-HT has been shown to attenuate mechanical and cold hypersensitivity (Rahman et al., 2006), while a loss of spinal noradrenaline neurotransmission modulates mechanical, but not heat hypersensitivity (Rahman et al., 2008) in the SNL model. These data suggest that disruptions of tonic descending noradrenergic inhibitory control coupled with an enhancement of descending serotonergic facilitation may modulate and underlie enhanced pain responding following SNL. Thus, chronic AMI increases serotonergic and noradrenergic neurotransmission in spinal and supraspinal sites, compensating for the SNL-induced changes in this system and preventing thermal hypersensitivity. The distinct effect of this pharmacological agent in OB-SNL vs. sham-SNL animals may be a result of altered monoaminergic neurotransmission following removal of the olfactory bulbs (for review see Kelly et al., 1997, Song and Leonard, 2005), thus AMI interacts differentially with descending pathways and supraspinal regions in sham and OB animals following SNL. These data may have important implications for the treatment of chronic pain states in depression.

In comparison to the effect of chronic AMI treatment, chronic minocycline treatment attenuated SNL-induced mechanical allodynia in both sham and OB rats although the magnitude and onset of the treatment response was greater in OB animals. Furthermore, chronic minocycline also attenuated
cold allodynia in both sham and OB animals. While previous studies have shown that chronic minocycline prevents OB-induced hyperactivity in the open field (Borre et al., 2012b) and attenuates SNL-induced mechanical allodynia (Guasti et al., 2009), the current data demonstrate that inhibition of microglial activation attenuates SNL-induced mechanical and cold allodynia in the presence and absence of a depressive-like phenotype. We propose that the earlier onset of treatment response in OB animals may be due to priming of microglia in the CNS following bulbectomy, which are then further activated in response to SNL, giving a greater substrate on which minocycline can exert its therapeutic effect. The data presented here provide preventative efficacy for minocycline and evidence for microglial activation in the interaction between depression and pain.

Thus, amitriptyline and minocycline both attenuate depressive-like behaviour in the OB rat without effect on basal nociceptive responding, and both attenuate SNL-induced mechanical allodynia in the OB model. However, minocycline has a greater effect of attenuating mechanical allodynia in OB rats and also prevents SNL-induced cold allodynia. Although minocycline and amitriptyline work on different systems (microglia vs. serotonin/noradrenaline), both share common pathways, for example, inhibition of nitric oxide (Lin et al., 2001, Farghaly et al., 2012) and increasing glutamate reuptake (Tai et al., 2006, Nie et al., 2010). In comparison, these treatments also differentially regulate other substrates. For example, chronic minocycline but not amitriptyline treatment reduces BDNF in the spinal cord following nerve injury (Vanelderen et al., 2013), whereas amitriptyline (Hu et al., 2010) but not minocycline (Arakawa et al., 2012) enhances BDNF in the hippocampus. Thus, effects such as these may account for similar outcomes on depressive-like behaviour but differential effects on neuropathic pain responding.

When we examined monoamine levels in the brain following chronic AMI, we failed to observe significant changes in these neurotransmitters despite behavioural efficacy of AMI in attenuating depressive-like behaviour and SNL-induced mechanical allodynia (Chapter 4). AMI may have altered
neurotransmission at receptor, transporter or signalling levels that were not assessed in the current thesis. However, taken together there appears to be a role for this system in behavioural modulation of affect, pain and the interaction between these conditions.

In addition, it has been shown that minocycline protects against degeneration of monoaminergic neurons via inhibition of neuroinflammation (Wixey et al., 2011, Zhang et al., 2006a, Orio et al., 2010). Thus, glial activation occurs upstream to monoaminergic deficits and as such prevention of microglial activation and subsequent neurodegeneration may account for the greater efficacy of minocycline seen in the current thesis. Therefore, microglia may be a preferred target for depression and chronic pain.

7.5 Differential neuroimmune mRNA expression in response to chronic antidepressant and anti-inflammatory treatment in the presence of a depressive-like phenotype

We examined the effect of chronic amitriptyline and minocycline treatment on the expression of inflammatory mediators in the brain. The OB-associated increase in GFAP and IL-10 mRNA expression in the PFC were not seen following AMI treatment, suggesting that chronic antidepressant treatment can normalise the increases in inflammatory gene expression associated with the combination of depression (OB) and neuropathic pain (SNL). In contrast, chronic minocycline did not alter GFAP expression in the PFC of OB-SNL animals, but rather enhanced IL-10 mRNA expression as well as IL-1β and IL-6. IL-10 injected into the midbrain has been shown to attenuate nerve injury-induced mechanical allodynia (Wang et al., 2012b) and elicit antidepressant effects in the forced swim test when administered i.c.v. to models of chronic stress (Pan et al., 2013, Voorhees et al., 2013). Thus, the enhancement of IL-10 by minocycline may account, at least in
part, for the antidepressant or anti-allodynic effects observed in Chapter 5. Taken together, AMI attenuates depressive-like behaviour and SNL-induced mechanical allodynia, and normalises GFAP and IL-10 mRNA in the PFC of OB-SNL animals. In contrast, chronic minocycline attenuates depressive-like behaviour, and SNL-induced mechanical and cold allodynia with a concurrent enhancement of IL-10, IL-1β and IL-6 mRNA expression in the PFC. These data indicate differential effects of a monoaminergic-based antidepressant and a microglial inhibitor on glia and cytokine mRNA expression in the PFC of OB animals following peripheral nerve injury.

While the data presented greatly enhance our knowledge on possible neurobiological mechanisms underlying the affective modulation of neuropathic pain, it should be noted that there are some limitations to the findings. In particular, due to methodological constraints and the requirement for a large number of animals, the expression of immune modulators and monoamines could only be examined at one time point, thus providing a snapshot of possible changes within certain brain regions. In addition, due to the multiple number of cytokines and levels of monoamines assessed in the tissue samples, there was not sufficient tissue remaining to examine protein levels of the immune mediators, as mRNA changes are not always translated to protein. However, pharmacological probing allowed us to examine the role of these systems in depressive-like, nociceptive and neuropathic pain behaviour in greater detail and at several time points. It should also be noted that chronic treatment with minocycline and amitriptyline was started prior to/on the day of OB surgery and such a treatment strategy may be analogous to a pretreatment regime. However, previous data has demonstrated an antidepressant effect of chronic AMI when initiated from 8 - 14 days post-OB (Mar et al., 2000, Lumia et al., 1992, van Riezen et al., 1977). Thus, OB-induced depressive-like behaviour can be attenuated by chronic antidepressant treatment beginning immediately following or after establishment of the OB syndrome.

Based on the preclinical evidence, including that presented in this thesis, and anecdotal clinical evidence, there may be some potential for the use of
minocycline in the prevention of the development of neuropathic pain following surgery, amputation, diabetes, chemotherapy etc., particularly in patients who have already been diagnosed with depression, thus having a higher risk of developing a chronic pain condition. Moreover, depressed patients on successful antidepressant (e.g. amitriptyline) treatment may have a lower risk of developing chronic pain. With this in mind, the data provided within form a foundation upon which to design further mechanistic and translational studies aimed at elucidating the neural substrates and neuroimmune mechanisms underpinning depression, early life stress and pain.

In conclusion, the work presented herein adds to the body of knowledge reporting altered nociceptive responding in mood disorders and provides a possible monoaminergic and neuroimmune basis for the interaction between depression and pain.


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