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Altered neuropathic pain behaviour in a rat model of depression is associated with changes in inflammatory gene expression in the amygdala

Running head: *Altered neuropathic pain behaviour in OB rats*

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Abbreviations: contra Contralateral; CD11b cluster of differentiation molecule 11b; GFAP Glial fibrillary acidic protein; IL-1 β Interleukin-1 beta; ipsi Ipsilateral; NSNL Non-Spinal Nerve Ligated; OB Olfactory Bulbectomy; SNL Spinal Nerve Ligation; TNF α Tumour necrosis factor alpha.

Abstract

The association between chronic pain and depression is widely recognised, the co-morbidity of which leads to a heavier disease burden, increased disability and poor treatment response. The present study examined nociceptive responding to mechanical and thermal stimuli prior to and following L5-L6 spinal nerve ligation (SNL), a model of neuropathic pain, in the olfactory bulbectomised (OB) rat model of depression. Associated changes in the expression of genes encoding for markers of glial activation and cytokines were subsequently examined in the amygdala, a key brain region for the modulation of emotion and pain. OB rats exhibited mechanical and cold allodynia, but not heat hyperalgesia, when compared to sham-operated counterparts. SNL induced characteristic mechanical and cold allodynia in the ipsilateral hind-paw of both sham and OB rats. 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, respectively. SNL reduced IL-6 and increased IL-10 expression in the amygdala of sham rats. The expression of CD11b and GFAP, indicative of microglial and astrocyte activation, and IL-1 β in the amygdala was enhanced in OB animals when compared to sham counterparts, an effect not observed following SNL. The present study demonstrates that neuropathic pain-related responding to an innocuous cold stimulus is altered in an animal model of depression, effects accompanied by changes in the expression of neuroinflammatory genes in the amygdala.

Introduction

Clinical co-morbidity of depression and pain is widely recognised, with over 50% of chronic pain patients experiencing depression, while patients with depression are over twice as likely to develop chronic pain (Bair *et al.*, 2003, Gameroff & Olfson, 2006). Animal models provide an important means of understanding the neurobiological basis of depression-pain co-morbidity. Depressive-like behaviour has been observed in several animal models of neuropathic pain (Fukuhara *et al.*, 2011, Hu *et al.*, 2009, Suzuki *et al.*, 2007, Wang *et al.*, 2011). Conversely, reserpine-induced monoamine depletion in rats elicits both depressive-like behaviour and mechanical allodynia (Arora *et al.*, 2011, Nagakura *et al.*, 2009). Chronic mild stress, chronic restraint stress and Wistar-Kyoto rat models of depression exhibit hyperalgesia to formalin and complete Freund's adjuvant, models of persistent inflammatory pain (Bardin *et al.*, 2009, Shi *et al.*, 2010b, Wang *et al.*, 2012). Furthermore, mechanical allodynia following peripheral nerve injury is enhanced in Wistar-Kyoto rats (Zeng *et al.*, 2008) and following chronic restraint stress (Norman *et al.*, 2010b) or social isolation (Norman *et al.*, 2010a). The olfactory bulbectomised (OB) rodent is a well-validated animal model of depression (Willner & Mitchell, 2002), which exhibits behavioural, neurotransmitter, neuroendocrine and immune changes resembling those reported clinically (Kelly *et al.*, 1997, Song & Leonard, 2005). 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 & Leonard, 2005). Recent studies have demonstrated that OB rats exhibit mechanical allodynia, enhanced formalin-evoked inflammatory pain (Burke *et al.*, 2010, Su *et al.*, 2010) and increased pain responding to electrical stimulation of the dura mater (Liang *et al.*, 2011). However, neuropathic pain responding has not been evaluated in the model, and therefore, the effect of bulbectomy on

nociceptive responding to mechanical and thermal stimuli prior to and following spinal nerve ligation (SNL) was evaluated in the current study.

Neuroinflammatory processes are well-recognised to play important roles in the pathophysiology of both depression and chronic pain (Miller *et al.*, 2009, Panigada & Gosselin, 2011, Watkins & Maier, 2005). For example, central IL-1 β plays a key role in chronic stress-induced depressive behaviour (Goshen *et al.*, 2008, Koo & Duman, 2008), IL-6 in the amygdala increases immobility in the forced swim test (Wu & Lin, 2008), chronic stress exposure increases IL-1 β production specifically in the amygdala (Porterfield *et al.*, 2012) and repeated psychosocial stress increases microglial activation in the amygdala (Wohleb *et al.*, 2011). Spinal inflammatory processes are essential for the development of neuropathic pain (Vallejo *et al.*, 2010), however, the role of neuroimmune mediators in supraspinal sites such as the amygdala is less understood. Prostaglandin E2 production is increased in the amygdala in a postoperative pain model (Shavit *et al.*, 2006), and recent data have indicated a role for TNF α in the amygdala in anxiety and persistent inflammatory pain (Chen *et al.*, 2013). Reserpine-induced depression-pain syndrome is associated with enhanced central inflammatory cytokines (Arora *et al.*, 2011) and central administration of IL-1ra ameliorates the effects of neuropathic pain on depressive behaviour (Norman *et al.*, 2010b). Thus, glial activation and cytokines in key brain regions such as the amygdala are involved in both affective and nociceptive processing, and may be responsible for the altered nociceptive responding associated with depression. As such, a further aim of this study was to determine if interactions between OB and SNL at a behavioural level are associated with concomitant alterations in the expression of genes encoding for neuroimmune mediators in the amygdala.

Material and Methods

Animal husbandry

Male Sprague Dawley rats (Charles River, Margate, UK) weighing 175-200g on arrival were singly housed in plastic-bottomed cages (45 × 25 × 20 cm) containing wood shavings as bedding, in a temperature controlled room (21 ± 1°C), with a 12:12h light-dark cycle (lights on at 0700h). Rats were singly housed and 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. All efforts were made to minimize the number of animals used, and their suffering.

Experimental design

The experimental design is presented in Figure 1. Essentially, animals were tested in the open field, von Frey test, Hargreaves test and the acetone drop test in order to determine baseline locomotor and nociceptive responding, following which animals were randomly assigned to either sham or olfactory bulbectomy (OB) surgery groups. Two weeks following surgery, animals were re-tested in the aforementioned tests. Animals were subsequently 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). Mechanical allodynia was examined on days 1, 5, 8, 12 and 15 following SNL or NSNL surgery and locomotor activity was re-examined on day 14. Animals were tested in the acetone drop test and Hargreaves test on day 19 and 20 post SNL/NSNL, respectively. Twenty-four hours following the last behavioural

assessment, animals were sacrificed by decapitation and the amygdala dissected out rapidly on an ice-cold plate and stored at -80°C until quantitative RT-PCR analysis was performed for the expression of inflammatory mediators. The genes selected for analysis included cluster of differentiation molecule 11b (CD11b; a marker of microglial activation); glial fibrillary acidic protein (GFAP; a marker of astrocyte activation); the pro-inflammatory cytokines, interleukin-1 beta (IL-1 β), IL-6 and tumour necrosis factor alpha (TNF α) and the anti-inflammatory cytokine IL-10.

Olfactory bulbectomy (OB) surgery

Bilateral olfactory bulbectomy (OB) was performed on rats anaesthetized with isoflurane (Abbot Laboratories, Berkshire, UK, [3% induction, 1.5% maintenance in 0.5L/min O₂]) as outlined previously (Burke *et al.*, 2010, Roche *et al.*, 2007; 2008). In brief, following the application of local anaesthetic (bupivacaine HCl 0.25%), the head was shaven and a midline sagittal incision was made in the skin overlying the skull. Two burr holes of 2mm diameter were drilled into the skull 5mm rostral to bregma and 2mm lateral to the midline. The olfactory bulbs were removed by gentle aspiration with a blunt hypodermic needle attached to a water vacuum pump and care was taken not to damage the frontal cortex. The burr holes were then plugged with a haemostatic sponge (Lohans Pharmacy, Galway, Ireland) to control bleeding. Sham-operated animals were treated in the same manner but the bulbs were left intact. Animals were handled daily following surgery and lesions were verified by gross inspection after 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.

L5-L6 Spinal nerve ligation (SNL) surgery

L5-L6 Spinal nerve ligation (SNL) is a well-characterised model of chronic neuropathic pain and was carried out as described previously (Kim & Chung, 1992, Moriarty *et al.*, 2012). Briefly, the rats were anaesthetized with isoflurane (2.5% in 0.6 L/min O₂), the fur lateral to the midline on the left-hand side at the lower lumbar and sacral regions was clipped and an incision was made through the skin between the spinal column and the left iliac crest. Paraspinal muscles were removed using a toothed forceps to visualise the L6 transverse process, which was removed and the L5 and L6 nerves were tightly ligated using 6-0 silk suture (Interfocus, Cambridge, UK). NSNL rats were treated in the same manner, however the L5 and L6 nerves were exposed but not ligated. Rats were allowed to recover from anaesthesia in heated recovery cages and subsequently returned to their home cage.

Behavioural Testing

Open Field Test

Open field behavioural testing was carried out the day prior to sham or OB surgery, on day 14 post sham/bulbectomy, and on day 14 post SNL/NSNL surgery. Exposure of OB rats to a novel open field arena results in a hyperactive locomotor response, a hallmark of depressive-like behaviour in the model which is selectively reversed by chronic, but not acute, antidepressant treatment (Kelly *et al.*, 1997, Song & Leonard, 2005). As such, locomotor activity was assessed over a 5 minute period in the open field to confirm OB-induced hyperactivity 14 days post surgery and to confirm that the depressive-like phenotype was maintained following SNL/NSNL surgery. On the experimental day, each animal was removed from the home cage during the light phase between 1000h and 1200h and placed singly into a brightly lit (lux 300-400) novel open field arena (diameter 75cm) where locomotor activity (distance moved, cm) and time spent (s) in the inner zone (diameter

55cm) was assessed using a computerised video tracking system (EthoVision®, Version 3.1, Noldus, Wageningen, Netherlands).

von Frey test

The arena used for von Frey testing consisted of a six-compartment Perspex arena (11cm × 20cm × 15cm) with wire mesh flooring. Rats were habituated to the arena for 20 minutes prior to testing, following which von Frey filaments (Touch-Test ®Sensory Evaluators, North Coast Medical, Inc., CA, USA) of different forces (0.16g – 100g) were used to determine the 50% withdrawal threshold as previously described (Moriarty *et al.*, 2012). Briefly, filaments were applied perpendicular to the plantar surface of the hind-paw, 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 of increasing force were applied to both left and right hind-paws five times (alternating between paws) until a 100% positive response (5 positive responses to 5 applications) was observed. The filament force eliciting a 50% response was calculated by plotting the percentage response versus filament force for each rat. von Frey testing was carried out prior to and 17 days following OB/sham surgery and on days 1, 5, 8, 12, and 15 post-SNL/NSNL surgery by an experimenter blind to the treatment procedure.

Acetone drop Test

The acetone drop test was used to measure responding to an innocuous cold stimulus as previously described (Moriarty *et al.*, 2012). Animals were placed in individual chambers on an elevated mesh floor and allowed to habituate for 20 minutes as for the von Frey test. Polyethylene tubing (2mm ID, Fisher Scientific, Dublin, Ireland) was attached to a 1ml syringe and used to apply approximately 0.2ml of acetone (Sigma-Aldrich, Dublin, Ireland)

to the plantar surface of the hind-paw. Latency to respond and withdrawal frequency (number of responses) within 60 seconds was recorded. 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, with at least a 3 minute interval.

Hargreaves Test

The Hargreaves apparatus (Plantar Analgesia Meter, IITC Life Science Inc., CA, USA) was used to measure thermal nociception and assesses heat hyperalgesia, as previously described (Moriarty *et al.*, 2012). Animals were placed in the apparatus on top of a glass panel heated to 30°C, and allowed to habituate for 20 minutes. A focused beam of radiant light (active intensity of 30%) was used to heat the plantar surface of the hind-paw 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 at least a 3 minute interval between testing.

Gene expression analysis using quantitative real-time PCR

RNA was extracted from amygdala tissue using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Düren, Germany). Genomic DNA contamination was removed with the addition of DNase to the samples according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, Warrington, UK). TaqMan gene expression assays (Applied Biosystems, Warrington, UK) containing forward and reverse primers and a FAM-labelled MGB TaqMan probe were used to quantify the gene of interest and real-time PCR was performed using an ABI Prism 7500 instrument (Applied Biosystems, Warrington, UK), as previously

described (Kerr *et al.*, 2012, Kerr *et al.*, 2013). Assay IDs for the genes examined were as follows: CD11b (Rn00709342_m1); GFAP (Rn00566603_m1); IL-1 β (Rn00580432_m1); TNF α (Rn99999017_m1); IL-6 (Rn00561420_m1) and IL-10 (Rn00563409_m1). PCR was performed using TaqMan Universal PCR Master Mix. The cycling conditions were 90°C for 10 minutes and 40 cycles of 90°C for 15 minutes followed by 60°C for 1 minute. β -actin was used as an endogenous control to normalise gene expression data. Relative gene expression was calculated using the $\Delta\Delta$ CT method and data expressed as % sham-NSNL controls.

Statistical analysis

PASW 18 statistical program was used to analyse all data. Data were assessed using either Student's unpaired t-test, Mann-Whitney U test, two-way analysis of variance (ANOVA) or repeated measures ANOVA to assess changes over time. Duncan's *post-hoc* analysis was performed following ANOVA where appropriate. Spearman's correlation analysis was used to assess the correlation between behavioural data and gene expression data. The level of significance was set at $P \leq 0.05$. All data were expressed as means \pm standard error of the mean (SEM).

Results

SNL induces anxiety-related behaviour in sham but not OB rats

OB animals demonstrated a characteristic hyperactivity response on exposure to the open field test 14 days following surgery, expressed as an increase in distance moved, when compared to sham-operated counterparts ($t_{34} = 5.02$ $P < 0.001$; Fig. 2a), an effect maintained following NSNL or SNL surgery (Two-way ANOVA effect of OB: $F_{(1,32)} = 4.44$ $P = 0.043$ Fig. 2b). Although OB rats exhibited a slight decrease in the amount of time spent in the inner zone, this effect failed to reach statistical significance, indicating no alteration in anxiety-related behaviour (Fig. 2b). Although SNL did not alter distance moved in the open field, it did reduce time spent in the inner zone in sham (sham-NSNL 47.53 ± 13.47 s vs. sham-SNL 14.75 ± 4.12 s; Two-way ANOVA effect of SNL: $F_{(1,31)} = 4.83$, $P = 0.036$; Fig 2b), but not OB, animals when compared to NSNL controls.

OB rats exhibit mechanical and cold allodynia, but not heat hyperalgesia

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 slight but non-significant increase in the withdrawal frequency ($U = 103.5$ $P = 0.06$) to the application of acetone to the hind-paws, when compared to sham-operated controls (Fig. 2b-c). In addition, OB rats displayed mechanical allodynia, demonstrated as a significant reduction in the 50% mechanical withdrawal threshold when compared to sham-operated controls ($t_{34} = 2.71$ $P = 0.011$, Fig. 2d). There was no significant effect of OB on the latency to respond to a noxious heat stimulus in the Hargreaves test (Fig. 2e).

OB rats exhibit altered SNL-induced behavioural responding to an innocuous cold stimulus

Following SNL surgery, both sham and OB rats exhibited a significant decrease in mechanical withdrawal thresholds of the ipsilateral hind-paw at all post-injury time points examined, when compared to pre-SNL thresholds (Repeated measures ANOVA effect of time $F_{(5,165)} = 12.12$ $P < 0.001$, time \times side interaction $F_{(5,165)} = 6.05$ $P < 0.001$; Fig 3a). SNL did not alter the OB-induced mechanical allodynia of the contralateral (right) hind-paw (Fig. 3a; Sham-contra vs. OB-contra). Due to the differences in nociceptive responding of sham and OB rats prior to SNL, behavioural data obtained following SNL were expressed as a percentage of pre-SNL values.

SNL induced mechanical and cold allodynia, but not heat hyperalgesia, of the ipsilateral hind-paw of both sham and OB rats (Fig. 3 b-c when compared to the dotted line representing pre-SNL levels). OB rats exhibited reduced paw withdrawal latency ($t_{20} = 2.76$ $P = 0.012$) and withdrawal frequency ($t_{19} = 3.21$ $P = 0.005$) to application of acetone to the ipsilateral hind-paw following SNL, when compared to sham-SNL controls, indicating altered nociceptive responding to a cold stimulus (OB-SNL vs. sham-SNL, Fig. 3b). There was no significant difference between sham and OB rats in the response threshold to mechanical, cold or heat stimuli of the contralateral hind-paw following SNL (Fig. 3c).

OB and SNL induced changes in gene expression of inflammatory mediators in the amygdala

Analysis of inflammatory mediator expression in the right and left amygdala revealed no significant lateralisation effects, thus data were pooled for subsequent analysis. OB resulted in increased mRNA expression of CD11b (marker of microglial activation), GFAP (marker of astrocyte activation) and the pro-inflammatory cytokine IL-1 β , in the amygdala when compared to sham counterparts (sham-NSNL vs. OB-NSNL, Fig. 4a-c), an effect attenuated in the presence of SNL (OB-NSNL vs. OB-SNL; Two-way ANOVA OB \times SNL interactions CD11b: $F_{(1,27)} = 4.22$ $P = 0.05$; GFAP: $F_{(1,27)} = 5.77$ $P = 0.023$; IL-1 β : $F_{(1,24)} = 4.50$ $P = 0.04$). There was no effect of OB or SNL on TNF α expression, however, SNL resulted in

reduced expression of IL-6 (SNL: $F_{(1,23)} = 21.4$ $P < 0.01$) in both sham and OB animals, and increased expression of IL-10 (SNL: $F_{(1,24)} = 10.56$ $P = 0.003$) in sham, but not OB, animals (Fig. 4e-f).

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 ($r = 0.620$, $P = 0.006$, Fig. 4g). The percentage change in 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 ($r = 0.381$, $P = 0.046$, Fig. 4h). There were no further significant correlations between behavioural responding and gene expression of inflammatory mediators.

Discussion

The present study demonstrates that the OB rat model of depression displays mechanical and cold allodynia, and altered neuropathic pain behaviour to an innocuous cold stimulus following SNL surgery. The reduced latency and increased number of responses in the acetone drop test following SNL surgery was positively correlated with IL-6 and IL-10 expression in the amygdala, respectively. Furthermore, the OB-associated increase in the expression of CD11b, GFAP and IL-1 β in the amygdala was not observed in SNL animals. This study provides a further preclinical model for studying the association between depression and neuropathic pain, and indicates that neuroimmune processes in the amygdala may, in part, underlie the behavioural changes observed.

Altered nociceptive responding in the OB model of depression

Prior to spinal nerve injury, OB rats exhibited mechanical allodynia, results consistent with those previously reported from our laboratory (Burke *et al.*, 2010), however to our knowledge, this is the first study to report that OB rats also exhibit cold allodynia. In comparison, nociceptive responding to heat stimuli was not altered in OB rats in the current study, although increased paw withdrawal latency to a radiant heat source and transient thermal heat hyperalgesia in the hot plate, but not tail flick, test have been previously reported in OB rats (Burke *et al.*, 2010, Su *et al.*, 2010, Wang *et al.*, 2010). Thus, OB animals appear to exhibit alterations in nociceptive responding to thermal stimuli depending on the test employed. The present findings correlate with those observed clinically where depressed patients exhibit decreased (Bar *et al.*, 2007, Lautenbacher *et al.*, 1994, Schwier *et al.*, 2010), increased (Chiu *et al.*, 2005, Gormsen *et al.*, 2004, Strigo *et al.*, 2008) 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 bulbectomy on neuropathic pain responding, demonstrating that OB rats exhibit altered nociceptive responding to an innocuous cold, but not heat or mechanical, stimulus following SNL. Specifically, OB-SNL rats exhibited reduced latency to respond and reduced number of responses of the ipsilateral hind-paw to an innocuous cold stimulus. We propose that the reduction in response latency may reflect enhanced initial perception of cold stimuli while the reduction in the number of responses might reflect a concomitant reduction in the duration or magnitude of the response in OB-SNL animals. The mechanisms mediating these alterations remain to be determined; however, it is possible that bulbectomy may result in disruption of nociceptive gating and descending pain pathways. Thus, following nerve injury, OB animals may exhibit enhanced gating of cold stimulus-related nociceptive information (reduced latency), however following initial perception, OB animals may be capable of engaging the descending inhibitory pain pathway more effectively, resulting in reduced magnitude or severity (number of responses) to the stimulus. It is possible that functional alterations in key brain regions part of the descending pain pathway such as the amygdala, and changes in glutamatergic, serotonergic or noradrenergic neurotransmission (Kelly *et al.*, 1997, Song & Leonard, 2005) may underlie the enhanced initial perception and/or the decrease in the magnitude of the SNL-induced cold allodynia in OB rats. This idea is discussed further below in the context of the OB-related changes in IL-6 and IL-10 expression observed herein. In contrast to our findings here, SNL and partial sciatic ligation result in a paradoxical increase in mechanical and thermal (heat) thresholds in the unpredictable chronic mild stress (Shi *et al.*, 2010a) and the Flinders sensitive line (Shir *et al.*, 2001) models of depression, respectively. Thus, differential neurobiological mechanisms may underlie the effects of unpredictable chronic mild stress and OB on nociceptive responding following SNL.

In accordance with previous studies (Suzuki *et al.*, 2007, Yalcin *et al.*, 2011), SNL induced anxiety-related behaviour in sham rats in the open field test, exemplified by the reduction in time in the inner area. In comparison, OB-SNL rats did not exhibit anxiety-related behaviour but maintained the OB-induced increase in locomotor activity on exposure to the open field. Although it cannot be ruled out that SNL may induce anxiety-related behaviour in OB animals at times or in paradigms other than those examined in the current study, the present data indicate that OB rats exhibit resilience to SNL-induced anxiogenesis in the open field.

Gene expression changes in the amygdala associated with altered nociceptive responding in the OB model

Limbic regions such as the amygdala play key roles in the processing of emotion and pain, and inflammatory mediators in discrete brain regions modulate both affective and nociceptive processing. Thus, neuroimmune processes in regions such as the amygdala may be the driving force for altered nociceptive responding associated with depression. Previous studies have demonstrated that OB rats exhibit increases in TNF α and/or IL-1 β levels in the prefrontal cortex, hippocampus and hypothalamus (Borre *et al.*, 2012, 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). To our knowledge, this is the first study to investigate the effect of bulbectomy on the expression of immune mediators in the amygdala, demonstrating enhanced gene expression of CD11b and GFAP, markers of microglial and astrocyte activation respectively, and of the proinflammatory cytokine IL-1 β , in the amygdala of OB rats. OB-induced hyperactivity has been shown to be associated with neurodegeneration within the amygdala (Jarosik *et al.*, 2007, Wrynn *et al.*, 2000), effects which may result from neuroinflammatory processes in this region following removal of the bulbs. In addition, increased glial activation and IL-1 β 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-1 β results in thermal (Oka *et al.*, 1993) and mechanical (Yabuuchi *et al.*, 1996) hyperalgesia. Interestingly, the increase in the expression of IL-1 β in the amygdala of OB rats was not observed following SNL, suggesting that spinal nerve injury can attenuate the increases in amygdaloid IL-1 β expression that result from central injury (OB). Indeed, SNL induced a decrease in IL-6 and an increase in IL-10 expression in the amygdala. The lack of effect of SNL on the expression of CD11b, GFAP or other proinflammatory cytokines (IL-1 β and TNF α) may not be surprising given the time at which these were examined post surgery (day 22). Previous studies have shown that GFAP, IL-1 β and TNF α protein levels are increased in the brain between day 3-7 post surgery (Liu *et al.*, 2007, Marcello *et al.*, 2013, Xie *et al.*, 2006). In comparison, IL-10 protein levels in the brain have been reported to increase over time following SNL, with highest levels observed 21 days post surgery (Xie *et al.*, 2006), correlating with the present findings.

Latency to respond to a cold thermal stimulus was positively 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. The number of responses to the 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 (Bachis *et al.*, 2001, Strle *et al.*, 2002) and thus changes in IL-10 expression may alter neuronal integrity and function within the amygdala. Such changes may increase descending inhibitory pain regulation and/or reduce descending facilitatory pain modulation, resulting in reduced

magnitude or severity (number of responses) to the cold stimulus as observed in OB-SNL rats.

In conclusion, the present findings demonstrate that in the presence of a depressive-like phenotype, neuropathic pain behaviour is altered depending on stimulus modality, effects accompanied by alterations in inflammatory mediator gene expression within the amygdala. Increased understanding of the neurobiological substrates underlying depression, chronic pain and the interaction between these disorders may provide novel therapeutic targets for treating these debilitating co-morbid disorders.

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Figure Legends

Fig 1: Experimental Protocol

Abbreviations: OB Olfactory bulbectomy, SNL Spinal Nerve Ligation, NSNL non-Spinal Nerve Ligation.

Fig. 2: Locomotor activity and nociceptive responding of sham and OB rats to mechanical and thermal (heat and cold) stimuli

(a) Distance moved in the open field of sham and OB animals 14 days post surgery, (b) Distance moved and time in the inner area of the open field of sham and OB animals following NSNL or SNL surgery, (c) paw withdrawal latency and (d) withdrawal frequency (no. of responses) following the application of acetone to the hind-paws, (e) mechanical withdrawal thresholds of the hind-paws to von Frey mechanical stimulation and (f) latency to withdraw from a noxious heat stimulus (Hargreaves test) applied to the hind-paws. Data expressed as mean \pm SEM, n = 18-20. *P < 0.05, **P < 0.01 vs. Sham-operated controls. OB Olfactory bulbectomy.

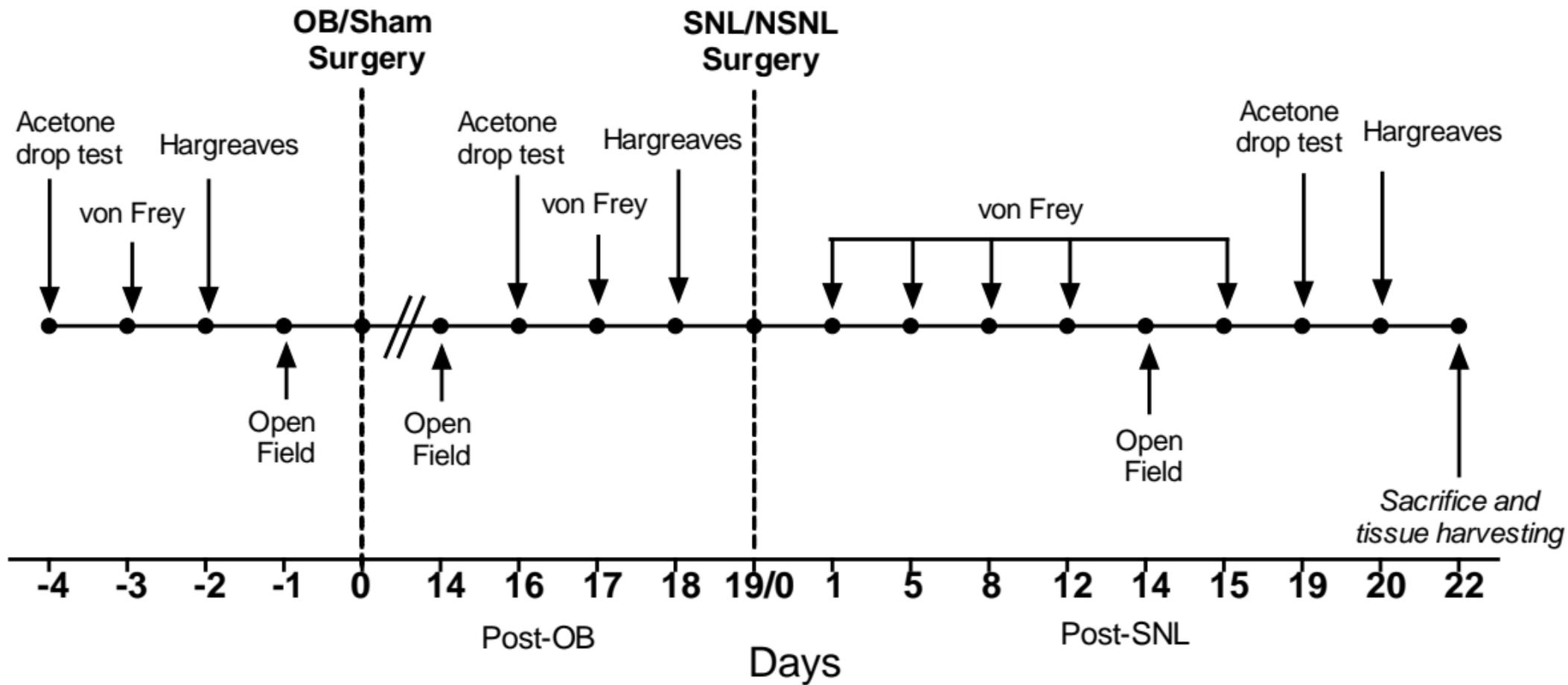
Fig 3: Nociceptive responding of sham and OB rats to mechanical and thermal (heat and cold) stimuli following SNL surgery

(a) Mechanical response thresholds of the ipsi- and contra-lateral hind-paw of sham and OB animals prior to and following SNL surgery (**P < 0.01 Sham-ipsi and OB-ipsi vs. pre-SNL levels, ⁺⁺P < 0.01 OB ipsi/contra vs. Sham-ipsi/contra). (b) Mechanical, cold and heat responding of the ipsilateral hind-paw of sham and OB rats following SNL expressed as percentage of pre-SNL values. Mechanical responding calculated as an average over post-SNL testing period (⁺P < 0.05, ⁺⁺P < 0.01 OB vs. sham, dotted line represents pre-SNL levels) (c) Mechanical, cold and heat responding of the contralateral hind-paw of sham and

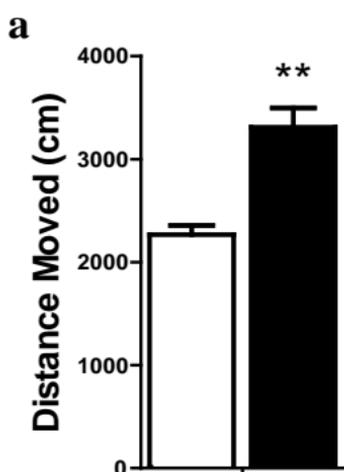
OB rats following SNL expressed as percentage of pre-SNL values. Data expressed as mean \pm SEM, n = 7-11. contra Contralateral; ipsi Ipsilateral; NSNL non-Spinal Nerve Ligation; OB Olfactory bulbectomy; SNL Spinal Nerve Ligation.

Fig. 4: Gene expression of neuroinflammatory mediators in the amygdala following OB and SNL.

The effect of OB, SNL and OB-SNL on the expression of (a) CD11b, (b) GFAP, (c) IL-1 β , (d) TNF α , (e) IL-6 and (f) IL-10. Data expressed as mean \pm SEM, n = 7-8. *P < 0.05 vs. sham-NSNL, #P < 0.05, ##P < 0.01 vs. OB-NSNL, +P < 0.05 vs. sham-SNL. &P < 0.05 overall effect of SNL (ANOVA). CD11b cluster of differentiation molecule 11b; GFAP Glial fibrillary acidic protein; IL-1 β Interleukin-1 beta; NSNL Non-Spinal Nerve Ligated; OB Olfactory Bulbectomy; SNL Spinal Nerve Ligation; TNF α Tumour necrosis factor alpha.



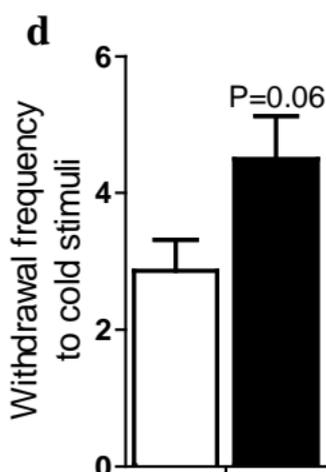
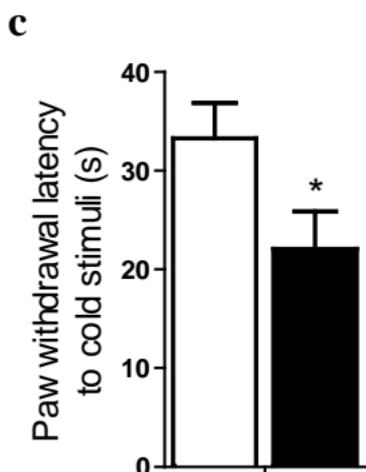
Sham
 OB



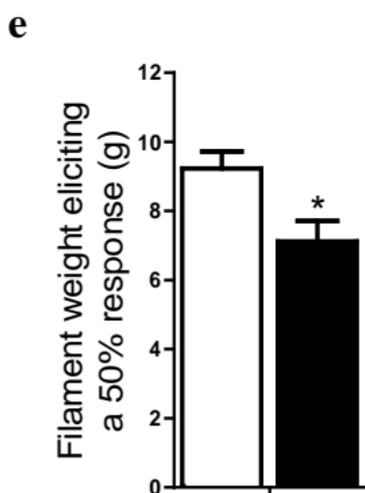
b

Distance moved (cm)		
	Sham	OB
NSNL	1792 ± 231	2156 ± 223 ^a
SNL	1713 ± 119	2210 ± 214 ^a
Time in the inner zone (s)		
NSNL	47 ± 13	19 ± 7
SNL	14 ± 4 [*]	18 ± 7

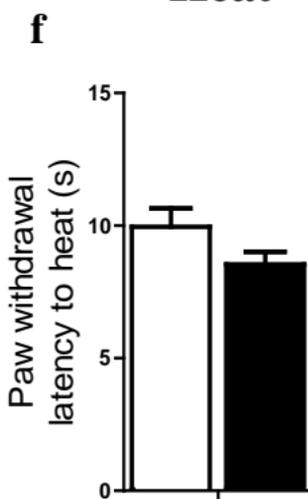
Cold

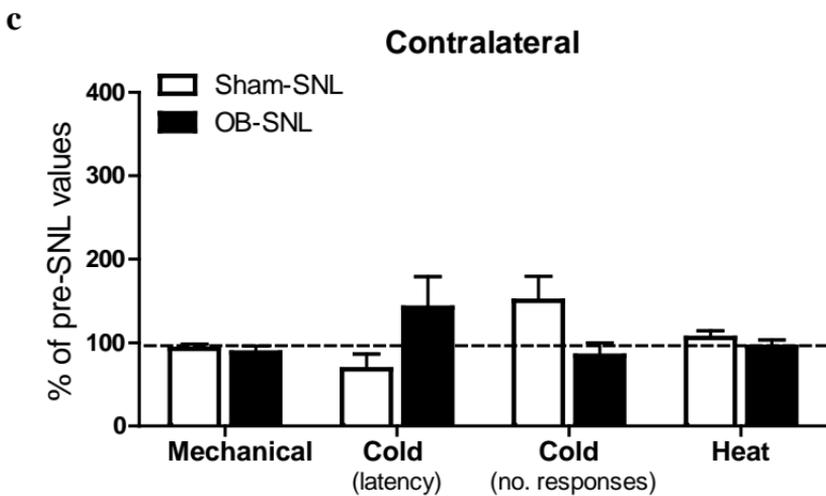
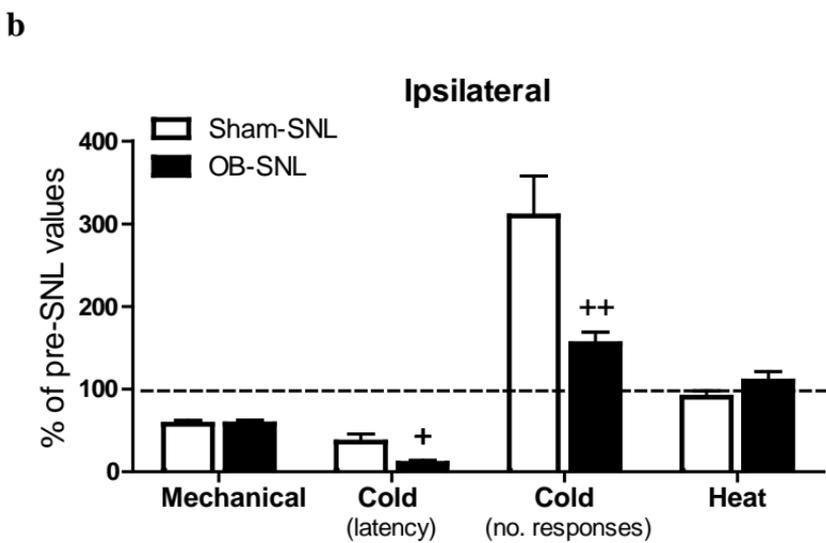
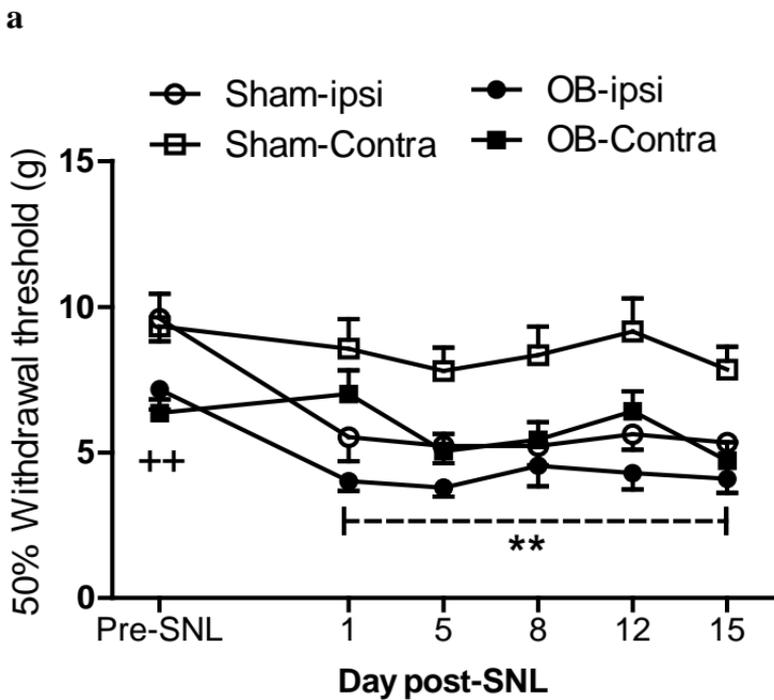


Mechanical



Heat





□ Sham
■ OB

