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Burke NN, Kerr DM, Moriarty O, Finn DP, Roche M (2014).

Minocycline modulates neuropathic pain behaviour and cortical M1-M2 microglial gene expression in a rat model of depression.

Brain, Behavior, and Immunity. 42: 147-156

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Keywords: olfactory bulbectomy, spinal nerve ligation, cytokine, microglia, prefrontal cortex, von Frey, allodynia, neuroinflammation, antidepressant

Abstract

There is a paucity of data on the role of microglia and neuroinflammatory processes in the association between chronic pain and depression. The current study examined the effect of the microglial inhibitor minocycline on depressive-like behaviour, spinal nerve ligation (SNL)-induced mechanical and cold allodynia and associated changes in the expression of genes encoding microglial markers (M1 vs. M2 polarisation) and inflammatory mediators in the prefrontal cortex in the olfactory bulbectomised (OB) rat model of depression. Acute minocycline administration did not alter OB-induced depressive-like behaviour but prevented SNL-induced mechanical allodynia in both OB and sham rats. In comparison, chronic minocycline attenuated OB-induced depressive-like behaviour and prevented the development of SNL-induced mechanical allodynia in OB, but not sham, rats. Further analysis revealed that SNL-induced mechanical allodynia in OB rats was attenuated by chronic minocycline at almost all time-points over a 2 week testing period, an effect observed only from day 10 post-SNL in sham rats. Chronic administration of minocycline reduced the expression of CD11b, a marker of microglial activation, and the M1 pro-inflammatory cytokine IL-1β, in the prefrontal cortex of sham-SNL animals. In comparison, the expression of the M2 microglia marker (MRC2) and anti-inflammatory cytokine IL-10 was increased, as were IL-1β, IL-6 and SOCS3, in the prefrontal cortex of OB-SNL animals following chronic minocycline. Thus, chronic minocycline attenuates neuropathic pain behaviour and modulates microglial activation and the central expression of inflammatory mediators in a manner dependent on the presence or absence of a depressive-like phenotype.

1. Introduction

Depression and chronic pain are widely recognised clinical co-morbidities, with up to 50% of chronic pain patients exhibiting depression (Radat et al., 2013), while almost 60% of depressed patients experience painful physical symptoms (Aguera-Ortiz et al., 2011). Recent data from animal studies support this clinical relationship and indicate that inflammatory and neuropathic pain-related responding is enhanced in various animal models of depression including olfactory bulbectomy (OB) (Burke et al., 2013a, Burke et al., 2010), early life stress (Uhelski and Fuchs, 2010, Burke et al., 2013b) and the Wistar-Kyoto rat (Burke et al., 2010, Zeng et al., 2008, Rea et al., 2014). However, the neurobiological substrates mediating altered nociceptive responding in depression remain ambiguous. Substantial evidence indicates dysregulation of the (neuro)immune system in both depression and chronic pain (for review see Miller et al., 2009, Watkins and Maier, 2005); which may also underlie the association between these conditions. Pre-clinical studies have demonstrated peripheral nerve injury-induced neuroimmune activation in brain regions responsible for the modulation of nociception and/or affect, including the prefrontal cortex (Apkarian et al., 2006, Al-Amin et al., 2011). Furthermore, the expression of genes coding for the pro-inflammatory cytokines IL-1β, IL-6 and/or TNFα, is enhanced in brain regions responsible for processing emotion and pain in animal models of depression with concomitant inflammatory (Kim et al., 2012, Arora et al., 2011) and neuropathic (Norman et al., 2010, Apkarian et al., 2006, Burke et al., 2013a, Burke et al., 2013b) pain-related responding. Thus, modulating neuroimmune responses has been proposed as a novel target for the treatment of CNS disorders with an inflammatory component, including depression and associated chronic pain.

Antidepressant efficacy of minocycline, a second-generation antibiotic that also inhibits microglial activation, has been reported both clinically (Miyaoka et al., 2012) and in animal models (Henry et al., 2008, O'Connor et al., 2009, Arakawa et al., 2012, Hinwood et al., 2012). Furthermore, minocycline has been shown to be beneficial in pain states such as rheumatoid arthritis (Langevitz et al., 2000) and sciatica (Sumracki et al., 2012). Both systemic and central administration of minocycline has been shown to prevent microglial activation and release of pro-inflammatory cytokines/mediators, and consequently the development of nerve injury-induced allodynia in several models (Raghavendra et al., 2003, Zanjani et al., 2006, Guasti et al., 2009, Pu et al., 2013, LeBlanc et al., 2011, Wei et al., 2008). However, it remains unknown what effect minocycline may have on neuropathic pain responding and associated supraspinal neuroimmune activity in the presence of a depressive-like phenotype.

Microglial activation can be divided into two phenotypic profiles: the *classical* pro-inflammatory M1 polarisation state, associated with increased release of pro-inflammatory cytokines such as IL-1β and TNFα; and the *alternative* anti-inflammatory M2 activation state, associated with the release of anti-inflammatory cytokines such as IL-10. Recent research has reported dynamic polarisation of microglia in models of ischemic stroke (Hu et al., 2012), spinal cord injury (for review see David and Kroner, 2011), and anxiety (Li et al., 2014). However, to date, microglial polarisation (M1 vs. M2) has not been examined in models of either depression and/or chronic pain.

The current study examined the effect of acute or chronic minocycline treatment on depressive-like behaviour and mechanical allodynia, prior to and following L5-L6 spinal nerve ligation (SNL), in the olfactory bulbectomised (OB) rat model of depression. Recent

data from our laboratory and others have demonstrated that OB rats exhibit mechanical allodynia, enhanced formalin-evoked nociceptive behaviour (Burke et al., 2013a, Burke et al., 2010, Wang et al., 2010), increased nociceptive behaviours to electrical stimulation of the dura mater (Liang et al., 2011), and altered nociceptive behaviour following SNL (Burke et al., 2013a). This latter effect has been shown to be associated with altered central expression of the cytokines IL-6 and IL-10 (Burke et al., 2013a). In order to test the hypothesis that supraspinal neuroimmune processes may underlie the antidepressant and anti-allodynic effect of chronic minocycline administration, the expression of markers of microglial polarisation and inflammatory cytokines were examined in the prefrontal cortex of sham and OB rats following SNL. The prefrontal cortex was chosen as it is a key brain region implicated in the interaction between depression and pain. Depressed patients exhibit increased activation of the prefrontal cortex in response to pain (Graff-Guerrero et al., 2008, Lopez-Sola et al., 2010, Bar et al., 2007), inhibition of prefrontal cortical activity enhances inflammatory hyperalgesia in the chronic mild stress rat model of depression (Qi et al., 2013), and the prefrontal cortex has been shown to exhibit altered neuroimmune activation/functioning in the OB model (Borre et al., 2012, Myint et al., 2007) and following nerve injury (Apkarian et al., 2006).

2. Materials and Methods

2.1 Animal husbandry

Male Sprague-Dawley rats (180-220g: Charles River, UK) were housed singly in plastic-bottomed cages containing wood shavings as bedding, in a temperature-controlled room (20 ± 2ºC), 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; 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.

2.2 Experimental design

Experiment 1: The effect of acute minocycline administration on locomotor activity and mechanical withdrawal thresholds, prior to and following SNL, in sham and OB rats

Systemic administration of minocycline has been shown to prevent the development of nerve-injury induced allodynia when administered prior to injury (Raghavendra et al., 2003, Zanjani et al., 2006), without affecting basal nociceptive responding (Mika et al., 2009, Padi and Kulkarni, 2008) or locomotor activity (Zhang et al., 2007). However, it is unknown if similar effects would occur in an animal model of depression. Thus, 2 weeks following sham or OB surgery, rats received minocycline [80mg/kg i.p.; Minocycline Hydrochloride, Hovione Ltd, Loures, Portugal; dose based on previous published literature demonstrating antidepressant effects at this dose (Molina-Hernandez et al., 2008)], or saline (2ml/kg) (4

groups: Sham-Veh [n=10], Sham-Mino [n=7], OB-Veh [n=9], OB-Mino [n=8]). Locomotor activity (open field) and mechanical withdrawal thresholds (von Frey test) were assessed 3 and 4 hours post administration, respectively. Immediately following nociceptive testing, L5-L6 SNL surgery was carried out on all animals, and mechanical withdrawal thresholds were re-examined 24 hours later.

Experiment 2: The effect of chronic minocycline intake on locomotor activity and nociceptive responding, prior to and following SNL, in sham and OB rats

This study examined the effect of a minocycline treatment regime that elicits an antidepressant-like effect in OB rats, on mechanical and cold allodynia, prior to and following SNL. Rats were provided with free access to either minocycline (1mg/ml) or tap water in their drinking bottle (route of administration and concentration based on previous work indicating microglial inhibition when administered chronically, Hinwood et al., 2012), beginning 24h prior to sham/OB surgery and continuing throughout the study (4 groups: Sham-Water [n=10], Sham-Minocycline (Mino) [n=11], OB-Water [n=11], OB-Mino [n=11]). Water bottles were weighed daily to determine fluid intake. Fresh drug stock was prepared and replaced every 3-4 days based on data indicating minimal degradation of minocycline in solution at room temperature over 7 days (Pearson and Trissel, 1993). Two weeks following sham/OB surgery, locomotor activity and nociceptive responding to mechanical and cold (acetone drop test: day 15) stimuli were assessed. SNL surgery was subsequently carried out on all animals and mechanical withdrawal thresholds were re-assessed 24hrs later. As differential effects of chronic minocycline were observed on nociceptive responding between sham and OB rats, mechanical withdrawal thresholds were reassessed on days 3, 7, 10 and 14 post-SNL and cold nociceptive responding assessed on day 14. Animals were

sacrificed by rapid decapitation, the prefrontal cortex dissected out, snap frozen and stored at -80^{o}C until assessment of cortical minocycline concentrations and inflammatory gene expression in the prefrontal cortex.

2.3 Bilateral Olfactory Bulbectomy (OB) surgery – model of depression

Bilateral olfactory bulbectomy (OB) was performed on rats anaesthetised with isoflurane (Abbot Laboratories, UK [3% induction, 1.5% maintenance in 0.5 l/min O_2]) as described previously (Roche et al., 2007, Burke et al., 2010, Burke et al., 2013a). In brief, two burr holes of 2mm diameter were drilled into the skull, 5mm rostral to bregma and 2mm lateral to the midline and the olfactory bulbs removed by gentle aspiration. The burr holes were plugged with a haemostatic sponge (Septodont, France) 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.

2.4 L5-L6 Spinal nerve ligation (SNL) surgery – model of neuropathic pain

SNL was carried out as described previously (Burke et al., 2013a, Kim and Chung, 1992, Moriarty et al., 2012). Briefly, the rats were anaesthetised with isoflurane (2.5% in 0.6 l/min O_2), the fur was clipped, an incision made between the spinal column and the left iliac crest and the paraspinal muscles removed. The L6 transverse process was removed and the L5 and L6 nerves were tightly ligated using 6-0 silk suture (Interfocus, UK).

2.5 Open Field Test

The open field test was used to confirm OB-induced hyperactivity, a hallmark of depressive-like behaviour in the model, and evaluate the anti-depressant-like activity of acute and chronic minocycline administration. OB-induced hyperactivity in the open field has been shown to be attenuated selectively following chronic but not acute, antidepressant administration (Roche et al., 2007, Song and Leonard, 2005, Kelly et al., 1997). Two weeks following sham/OB surgery, animals were placed singly into a brightly-lit (lux 200) novel open field arena (diameter 75cm) with a white floor (plastic-covered wood flooring) and reflective walls for a 5 minute period. Locomotor activity (distance moved, cm) was assessed using a computerised video tracking system (EthoVision®, Version 8 Noldus, Netherlands) as previously described (Burke et al., 2013a, Burke et al., 2010, Roche et al., 2007).

2.6 von Frey test for assessing mechanical allodynia

Rats were habituated to the testing arena (11cm × 20cm × 15cm) for 20 minutes prior to testing. von Frey filaments (Touch-Test® Sensory Evaluators, North Coast Medical, Inc., CA, USA) of different forces (0.07g – 100g) were used to determine 50% withdrawal thresholds, as previously described (Burke et al., 2013a, Moriarty et al., 2012). Briefly, filaments were applied to the plantar surface of the hind-paw, with sufficient force to cause 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 (five positive responses to five applications) was observed. 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.

2.7 Acetone drop test for assessing cold allodynia

The acetone drop test was carried 20 min following von Frey testing as previously described (Burke et al., 2013a, Moriarty et al., 2012). A drop of acetone (100%: Sigma-Aldrich, Ireland) was applied to the plantar surface of the hind-paw and latency to first response 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 with at least a 3 minute interval between testing the other paw. The average of the 4 trials was calculated for each hind-paw.

2.8 Gene expression analysis in the prefrontal cortex using quantitative real-time PCR

A High Capacity cDNA Archive kit (Applied Biosystems, UK) was used to reverse transcribe mRNA isolated using a NucleoSpin RNA II isolation kit (Macherey-Nagel, Germany) into cDNA. TaqMan gene expression assays (Applied Biosystems, UK) were used to quantify the genes of interest: cluster of differentiation molecule 11b (CD11b; Rn00709342_m1), CD68 (Rn01495634_g1), MRC2 (mannose receptor, Rn01456616_m1), Interleukin-1β (IL-1β; Rn00580432_m1), tumour necrosis factor-α (TNFα, Rn99999017_m1), IL-6 (Rn00561420_m1), IL-10 (Rn00563409_m1), SOCS3 (Rn00585674_s1) and IL-1 receptor antagonist (IL-1ra, Rn00573488_m1). RT-PCR was performed using an ABI Prism 7500 instrument (Applied Biosystems, UK), as previously described (Burke et al., 2013a, Kerr et al., 2013, Kerr et al., 2012, Burke et al., 2013b). β-actin (Rn00667869_m1) was used as an endogenous control and to normalise gene expression data. Relative gene expression was calculated using the $\Delta\Delta C_t$ method and data were expressed as % sham-SNL water-treated

controls.

2.9 Mass spectrometry determination of minocycline levels in cortical tissue

Cortical tissue samples (80-100mg) were sonicated in 400µl of homogenising buffer (4µg tetracycline/400µl 100% acetonitrile), homogenates centrifuged at 14,000g for 15 minutes at 4^{o}C and the supernatant collected and evaporated to dryness. 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). 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. Analyte detection was carried out in electrospray-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 concentration of minocycline was determined using ratiometric analysis with tetracycline as a standard of known concentration. Linearity was determined over a range of 3µg to 2.9ng. Results were expressed as µg/g tissue.

2.10 Statistical analysis

Parametric data were analysed using t-tests or two-way or repeated measures analysis of variance (ANOVA), followed by Fisher's LSD *post-hoc* testing where appropriate, using IBM SPSS 18 statistical program. Non-parametric data were analysed using Kruskal-Wallis followed by Mann-Whitney U *post-hoc* tests where appropriate. The level of significance was set at $P < 0.05$. All data are presented as the mean + SEM.

3. Results

3.1 Acute minocycline administration prevents the development of SNL-induced mechanical allodynia in sham and OB rats

OB rats exhibited a characteristic increase in locomotor activity upon exposure to a novel open field environment (OB effect: $F_{(1,29)}$=10.85, P=0.003, Fig. 1A) and a tendency to exhibit a decrease in mechanical withdrawal threshold in the von Frey test (OB effect: $F_{(1,27)}$=3.22, P=0.084, Fig. 1C) 14 days following surgery when compared to sham-operated counterparts. Acute minocycline administration did not alter locomotor activity or mechanical withdrawal thresholds of either sham or OB animals (Fig. 1A&C). SNL resulted in mechanical allodynia in both sham and OB rats, an effect prevented by acute minocycline pre-treatment (SNL×minocycline interaction: $F_{(1,26)}$=12.84, P=0.001, Fig. 1E).

3.2 Chronic minocycline attenuates OB-induced locomotor hyperactivity and prevents SNL-induced mechanical allodynia in OB but not sham animals

OB animals exhibited increased locomotor activity compared to their sham counterparts in the open field test 14 days following surgery (OB effect: $F_{(1,42)}$=15.486 P<0.001, Fig. 1B), an effect attenuated by chronic minocycline treatment (minocycline effect: $F_{(1,42)}$=6.45 P=0.015, Fig. 1B), indicative of an antidepressant-like effect in the model. OB animals exhibited a reduction in mechanical withdrawal thresholds when compared to sham-operated counterparts (OB effect: $F_{(1,41)}$=4.61 P=0.038, Fig. 1D), an effect not altered by chronic administration of minocycline. Following SNL, both sham and OB rats exhibited a reduction in mechanical withdrawal thresholds (SNL effect: $F_{(1,37)}$=6.55 P=0.015, Fig. 1E) when compared to pre-SNL thresholds. Chronic minocycline prevented the SNL-induced

decrease in withdrawal thresholds in OB, but not sham, animals (minocycline effect: $F_{(1,37)}$=4.18 P=0.048, Fig. 1E).

3.3 Chronic minocycline attenuates SNL-induced mechanical allodynia in both sham and OB animals with a differential temporal profile

Since chronic minocycline elicited an antidepressant-like effect and prevented the SNL-induced decrease in mechanical withdrawal thresholds in OB, but not sham, animals, we continued to evaluate nociceptive thresholds over a 2 week period post-SNL. Furthermore, as OB rats exhibited mechanical allodynia prior to SNL, and in order to compare directly across groups, data were expressed as a percentage of pre-SNL values. SNL resulted in a reduction in mechanical withdrawal thresholds of both sham and OB animals at all post-SNL time points when compared to pre-SNL levels (SNL effect: $F_{(5,175)}$=10.57 P<0.001, Fig. 2), indicative of SNL-induced mechanical allodynia. Chronic administration of minocycline attenuated the SNL-induced decrease in mechanical withdrawal thresholds of sham animals on day 10 and 14 post SNL when compared to water-treated counterparts (SNL×minocycline: $F_{(5,175)}$=6.38 P<0.001, Sham-SNL-Mino vs. Sham-SNL-Water). In comparison, chronic minocycline intake delayed the development of SNL-induced mechanical allodynia until day 3 in OB animals and attenuated allodynia from day 7 until 14 when compared to water-treated counterparts (SNL×OB×minocycline: $F_{(5,175)}$ = 3.46 P=0.005; OB-SNL-Mino vs. OB-SNL-Water).

3.4 Chronic minocycline treatment prevents SNL-induced cold allodynia in both sham and OB animals

Prior to SNL, OB rats exhibited a lower paw withdrawal latency to plantar application of acetone (cold) (OB effect: $F_{(1,42)}$=4.86, P=0.033, Fig. 3A) and tended to exhibit an increased withdrawal frequency (although not significant) when compared to sham-operated controls (OB-water vs. Sham-water), indicative of OB-related cold allodynia. Chronic minocycline intake did not alter latency to respond or the number of responses to plantar application of acetone in either sham or OB rats.

Re-examination of paw withdrawal latency and frequency to the application of acetone 14 days following SNL revealed that latency to respond was lower and withdrawal frequency was higher in both sham and OB-water treated animals when compared to pre-SNL levels, indicating SNL-induced cold allodynia (Fig.3 C-D). OB animals exhibited a slight reduction in latency to respond (which failed to reach statistical significance) and a significant increase in withdrawal frequency ($\chi^2_{(3)}$=20.07 P<0.001, sham-SNL-water vs. OB-SNL-water; Fig.3 C-D) following SNL, indicative of enhanced SNL-induced cold allodynia. Chronic minocycline intake attenuated the SNL-induced decrease in latency ($F_{(1,36)}$=54.39 P<0.001, Fig.3C) and increase in withdrawal frequency ($\chi^2_{(3)}$=20.07 P<0.001, Fig. 3D) in both sham and OB animals, signifying that chronic minocycline treatment attenuates SNL-induced cold allodynia.

3.5 Chronic minocycline intake decreases CD11b expression in the prefrontal cortex of sham, but not OB, rats

Although sham and OB rats consumed equivalent amounts of minocycline over the course of the study, evaluation of minocycline concentration in the brain at the end of the study revealed a slight, but non-significant (t_{13}=2.72 P=0.06), increase in minocycline levels in OB rats when compared to sham counterparts (Table 1). The effect of minocycline intake on microglial activation was evaluated by examining the gene expression of the microglial cell surface marker CD11b. Data revealed that chronic minocycline intake reduced CD11b expression in the prefrontal cortex of sham, but not OB, rats (OB×minocycline: $F_{(1,35)}$=4.22, P=0.047; Table 1).

3.6 Differential effects of chronic minocycline treatment on gene expression of inflammatory mediators in the prefrontal cortex of sham and OB rats following SNL

The expression of the M1 marker CD68 in the prefrontal cortex was increased in OB-SNL rats when compared to sham-SNL counterparts (OB effect: $F_{(1,37)}$=13.05, P<0.001, Fig. 4A), an effect not altered by chronic minocycline administration. Chronic minocycline administration reduced IL-1β (OB×minocycline: $F_{(1,37)}$=12.47, P=0.001) and SOCS3 (OB×minocycline: $F_{(1,37)}$=8.52, P=0.006) expression in sham-SNL animals, effects not observed in OB-SNL animals (Fig. 4C&H). In comparison, chronic minocycline administration increased the expression of the M2 microglial marker, MRC2 (OB×minocycline: $F_{(1,37)}$=4.84, P=0.034) and the associated anti-inflammatory cytokine IL-10 (minocycline effect: $F_{(1,34)}$=9.73 P=0.004) in the prefrontal cortex of OB-SNL animals when compared to water-treated counterparts. Furthermore, the expression of IL-1β (OBxminocycline: $F_{(1,37)}$=12.47, P=0.001) and IL-6 (OB×minocycline: $F_{(1,34)}$=5.53, P=0.025) were also increased in OB-SNL animals when compared to their water-treated counterparts (Fig.4).

4. Discussion

The data presented herein demonstrate that acute microglial inhibition with minocycline does not alter the OB-induced depressive-like behaviour but prevents the development of neuropathic pain-related behaviour in the presence and absence of a depressive-like phenotype. In comparison, chronic minocycline attenuates the OB-induced depressive-like behaviour and delays the development of SNL-induced mechanical allodynia until day 3 in OB rats. Furthermore, this treatment regime attenuated SNL-induced mechanical allodynia in OB rats on day 7, an effect not observed in sham rats until day 10 post SNL surgery. The behavioural effects of chronic minocycline were accompanied by differential changes in the expression of inflammatory mediators in the prefrontal cortex of sham and OB rats. Specifically, chronic administration of minocycline reduced the expression of the microglial activation maker CD11b and the M1 pro-inflammatory cytokine IL-1β in prefrontal cortex of sham-SNL animals. In comparison, chronic minocycline administration increased expression of the M2 microglial marker MRC2 and associated anti-inflammatory cytokine IL-10, as well as IL-1β and IL-6, in the prefrontal cortex of OB-SNL animals. Thus, chronic minocycline elicits differential effects on microglial polarisation and inflammatory mediators in the prefrontal cortex in the presence or absence of a depressive-like phenotype, effects which may underlie the treatment-related temporal changes in nociceptive responding in sham and OB rats following SNL.

4.1 Antidepressant-like effects of chronic minocycline in the OB rat

Antidepressant-like effects of minocycline have been demonstrated in several animal models (Henry et al., 2008, O'Connor et al., 2009, Hinwood et al., 2012, Arakawa et al.,

2012), with recent data expanding this to include the OB rat. Specifically, chronic minocycline has been demonstrated to attenuate OB-related hyperactivity, behavioural despair (forced swim test) and spatial memory deficits (Borre et al., 2012, Rinwa and Kumar, 2013). Our data confirm these findings, demonstrating that OB-induced hyperactivity on exposure to the open field is attenuated following chronic, but not acute, minocycline administration, mimicking the effects of other antidepressant agents in this model (Song and Leonard, 2005). The antidepressant-like effects of minocycline are proposed to be mediated via attenuation of microglial activation, reduction in pro-inflammatory mediators and neuroprotective mechanisms (Pae et al., 2008, Garrido-Mesa et al., 2013). Several studies from our group and others have demonstrated increased microglial activation, TNFα and/or IL-1β levels in the prefrontal cortex, hippocampus, amygdala and hypothalamus (Borre et al., 2012, Myint et al., 2007, Burke et al., 2013a) and neurodegeneration in the amygdala and hippocampus (Wrynn et al., 2000, Jarosik et al., 2007) of OB rats. The antidepressant-like effect of minocycline in OB rats is associated with an attenuation of increases in oxidative-nitrosative stress markers, TNFα and IL-6 levels and caspase 3 activity in the cortex and hippocampus (Rinwa and Kumar, 2013). Thus, minocycline may modulate microglial activation and neuronal reorganisation that occurs in response to removal of the bulbs, mitigating some of the pathological alterations observed in the model and resulting in an antidepressant-like effect.

4.2 Acute minocycline pretreatment prevents the development of neuropathic pain-related behaviour in both sham and OB animals

In accordance with previous published data from our group (Burke et al., 2010, Burke et al., 2013a), OB animals displayed enhanced nociceptive responding to both mechanical and cold

stimuli and enhanced cold allodynia following SNL. Neither acute nor chronic minocycline administration altered basal mechanical thresholds of either sham or OB rats. Similarly, studies have demonstrated that acute (Mika et al., 2009), repeated (3 days) (Yoon et al., 2012), or chronic (Guasti et al., 2009) minocycline administration does not alter nociceptive responding to mechanical stimuli in the absence of a noxious insult/nerve injury. Thus, microglial activation may not underlie the OB-related increase in nociceptive responding to sensory stimuli (prior to SNL), indicating a dissociation between depressive-like behaviour and allodynia in the absence of nerve injury. In comparison, microglial activation has been reported as essential for the development of neuropathic pain behaviour, while astrocytes are responsible for the maintenance of this chronic pain state (Vallejo et al., 2010). Acute pre-treatment with minocycline prevented the development of SNL-induced mechanical allodynia in sham rats, correlating with previous data in several nerve-injury models (Zanjani et al., 2006, Raghavendra et al., 2003, Pu et al., 2013), an effect also observed in OB rats. Thus, the data indicate that the inhibition of microglial activation immediately prior to nerve injury prevents the development of neuropathic pain-related behaviour in the absence and presence of a depressive-like phenotype.

4.3 Chronic minocycline treatment attenuates neuropathic pain-related behaviour in the OB rat

This study also aimed to address whether a minocycline treatment regime that elicits an antidepressant-like effect would modulate the development/expression of SNL-induced mechanical allodynia. Chronic minocycline treatment did not prevent the development of SNL-induced mechanical allodynia in sham animals, but delayed its onset until day 3 in OB rats. To our knowledge, this is the first study to examine the effect of long-term (2 week)

pre-administration of minocycline on nerve injury-induced allodynia, demonstrating differential effects versus acute pre-treatment. It is possible that the delay in SNL-induced mechanical allodynia in OB rats until day 3 may reflect a peak in the inflammatory cascade known to occur at this time point following SNL (Jin et al., 2003, O'Rielly and Loomis, 2006), which minocycline treatment is unable to prevent/attenuate. However, continued repeated minocycline intake attenuated SNL-induced mechanical allodynia in OB rats from day 7, an effect only observed in sham animals from day 10. Previous data have demonstrated that systemic administration of minocycline beginning 1 hour prior to surgery and daily thereafter attenuates SNL-induced mechanical allodynia from day 5 onwards (Guasti et al., 2009). Methodological differences such as route of administration (i.p. vs. p.o.), dose (30mg/kg/day vs. 76mg/kg/day), duration of treatment and prior surgery may account for the difference in time of onset of the anti-allodynic effects of minocycline between the two studies. Taken together, the data indicate that time-dependent anti-inflammatory and neuroprotective effects underlie the anti-allodynic effects of repeated minocycline treatment in response to nerve injury, with more rapid or alternative responses occurring in OB animals.

4.4 Chronic minocycline differentially alters microglial phenotype and cytokine expression in the prefrontal cortex of sham and OB rats following spinal nerve injury

Evidence suggests that the phenotype of activated microglia (M1 vs. M2) governs the repair and regeneration response following nerve injury (Kigerl et al., 2009, David and Kroner, 2011). M1 microglia are considered to be pro-inflammatory (releasing IL-1β, TNFα) and result in deleterious neuroinflammation while M2 (releasing IL-10, neurotrophins) are believed to promote neuroprotection and regeneration after injury (Olah et al., 2011, David

and Kroner, 2011, Jimenez et al., 2008). Although minocycline is well-regarded as an inhibitor of microglial activation, recent evidence indicates that minocycline selectively inhibits microglial M1, but not M2, polarisation both *in vitro* and in an animal model of amyotrophic lateral sclerosis (Kobayashi et al., 2013). Previous studies have shown that anti-inflammatory compounds (pentoxifylline, pioglitazone) attenuate neuropathic pain-related behaviour and related increases in M1 cytokines (TNFα, IL-1β) in the prefrontal cortex (Liu et al., 2007, Jia et al., 2010). This is the first study to examine if similar effects are observed with minocycline and reveal that chronic minocycline intake reduced the expression of CD11b, a marker of microglial activation, and the M1 cytokine IL-1β in sham-SNL rats. Thus, the anti-nociceptive effect of chronic minocycline on SNL-induced mechanical allodynia (in sham animals) is accompanied by reduced expression of M1 microglial processes in the prefrontal cortex. In contrast, the expression of the M1 cytokines (IL-1β and IL-6), the M2 microglial marker MRC2 and the anti-inflammatory cytokine IL-10, were increased in OB-SNL rats following chronic minocycline intake. Enhanced expression of M2 microglia and the anti-inflammatory cytokine IL-10 may serve to promote beneficial functions of microglia, such as phagocytosis, dampening inflammation and increase release of growth factors which are critical for repair and regeneration (Loane and Byrnes, 2010). Moreover, 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), while central IL-10 administration attenuates nerve-injury induced mechanical allodynia (Wang et al., 2012). Recent data has indicated that shifting of microglial polarisation to an M2 phenotype reduces spreading depression and possibly the initiation of migraine (Pusic et al., 2014). In addition to the well-known pro-inflammatory effect of IL-1β, this cytokine also

plays a role in CNS repair via the induction of glial-derived insulin-like growth factor 1 (Mason et al., 2001) and nerve growth factor (Lindholm et al., 1987). Taken together, chronic minocycline appears to differentially alter the expression of M1-M2 microglial markers/cytokines in the prefrontal cortex of SNL animals in the presence or absence of a depressive phenotype, an effect which may account, at least in part, for the behavioural effects observed.

Summary and Conclusion

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 microglial polarisation and inflammatory mediator expression in the prefrontal cortex of sham and OB animals following SNL. These data suggest a differential supraspinal mechanism of action of minocycline in the presence of a depressive-like phenotype, effects which may underlie its antidepressant and anti-allodynic effect following nerve injury. These data provide further evidence for a role for microglia in the pathogenesis of depression, the development of neuropathic pain, and the interaction between these disorders.

5. Funding and Disclosure

The authors would like to gratefully acknowledge funding received from the Discipline of Physiology; the Millennium Fund, National University of Ireland Galway and from the Higher Education Authority of Ireland under PRTLI4. N.B. received a College of Medicine, Nursing and Health Sciences, National University of Ireland Galway, Doctoral Fellowship. The authors declare no conflict of interest.

6. Acknowledgements

The authors would like to acknowledge technical assistance from Mr Brendan Harhen and Mr Siyuan Chen. The authors would like to thank David Lohan for sourcing and supply of minocycline.

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Table 1: Effect of minocycline intake on CD11b expression in the prefrontal cortex

	Sham-SNL-Water	Sham-SNL-Mino	OB-SNL-Water	OB-SNL-Mino
Minocycline intake (mg/kg/day)	-	76±2	-	71±1
Minocycline Conc. (µg/g tissue)	-	3.6±0.4	-	5.6±0.8
CD11b mRNA (%sham-SNL-Water)	100±9	72±6*	99±12	107±8

Both Sham and OB animals consumed equivalent amounts of minocycline on average per day over the course of the study. Minocycline levels were slightly, but not significantly, increased in the brain of OB vs. sham rats at the end of the study period. Chronic minocycline reduced CD11b expression in the prefrontal cortex of sham but not OB rats. *P<0.05 vs. Sham-SNL-water. Mino: minocycline, Conc: concentration, SNL: spinal nerve ligation; OB: Olfactory bulbectomy.

Fig. 1 Effect of acute and chronic minocycline administration on SNL-induced mechanical allodynia in sham and OB rats. Chronic (B) but not acute (A) minocycline administration attenuates the OB-associated increase in distance moved over 5 minutes in the open field test (Two-way ANOVA). (C-D) OB rats exhibit a decrease in mechanical withdrawal threshold (allodynia) when compared to sham-counterparts, an effect not altered by acute or chronic minocycline administration (Two-way ANOVA). (E) Acute minocycline administration prevents SNL-induced decreases in mechanical withdrawal thresholds in sham and OB animals (Repeated measures ANOVA). (F) Chronic minocycline administration prevents SNL-induced decreases in mechanical withdrawal thresholds in OB, but not sham, animals (Repeated measures ANOVA). Data expressed as mean ± SEM, n=7-12. *P<0.05 vs. Sham-veh/water, $^{+}$P<0.05 vs. Sham-mino, $^{\#}$P<0.05 vs. OB-Water, $^{\alpha}$P<0.05 effect of OB (ANOVA), $^{\delta}$P<0.05 $^{\delta\delta}$P<0.01 vs. pre-SNL values.

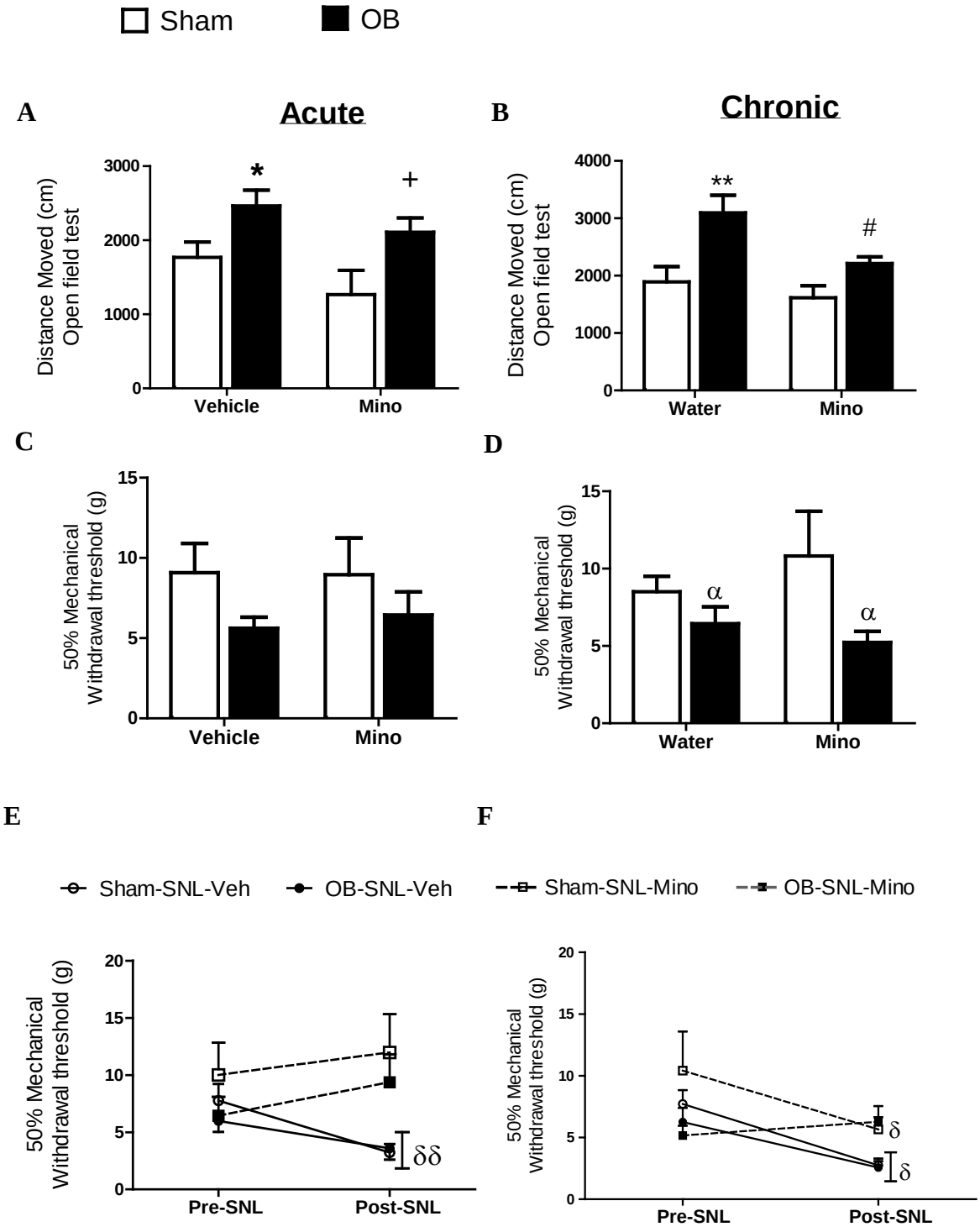
Fig. 2 Chronic minocycline induces differential effects on mechanical withdrawal thresholds following SNL in sham and OB rats. Data expressed as mean % change in withdrawal threshold from baseline (pre-SNL) ± SEM, n=10-12. (Repeated measures ANOVA) **P<0.05 vs. Sham-SNL-water, $^{\#\#}$P<0.01 vs. OB-SNL-Water.

Fig. 3 Effect of bulbectomy and chronic minocycline on nociceptive responding to an innocuous cold stimulus, prior to and following SNL. (A) Paw withdrawal latency (Two-way ANOVA) and (B) paw withdrawal frequency (Kruskal-Wallis) to plantar application of acetone prior to SNL. (C) Paw withdrawal latency (Two-way ANOVA) and (D) paw withdrawal frequency (Kruskal-Wallis) to application of acetone to the ipsilateral hind-paw 14 days following SNL. Dotted line represents pre-SNL average values. Data expressed as mean +

SEM. n=10-12. *P<0.05 vs. sham-water, **P<0.01 vs. sham-SNL-Water, $^{\#\#}$P<0.01 vs. OB-SNL-Water.

Fig. 4 Chronic minocycline intake elicits differential effects on microglial phenotypic markers and inflammatory mediators in the prefrontal cortex of sham and OB rats following SNL. Expression of microglial M1 phenotype markers (A) CD68 (B) TNFα (C) IL-1β (D) IL-6; M2 phenotype markers (E) MRC2 (F) IL-10; and cytokine signalling markers (G) IL-1ra and (H) SOCS3 in the prefrontal cortex. Data expressed as mean % change in expression from sham-SNL-Water treated animals + SEM, n= 10-12. (Two-way ANOVAs) *P<0.05 vs. sham-SNL-Water, $^{++}$P<0.01 vs. sham-SNL-Mino, $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. OB-SNL-Water.

Fig.1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Post-SNL

Fig 2

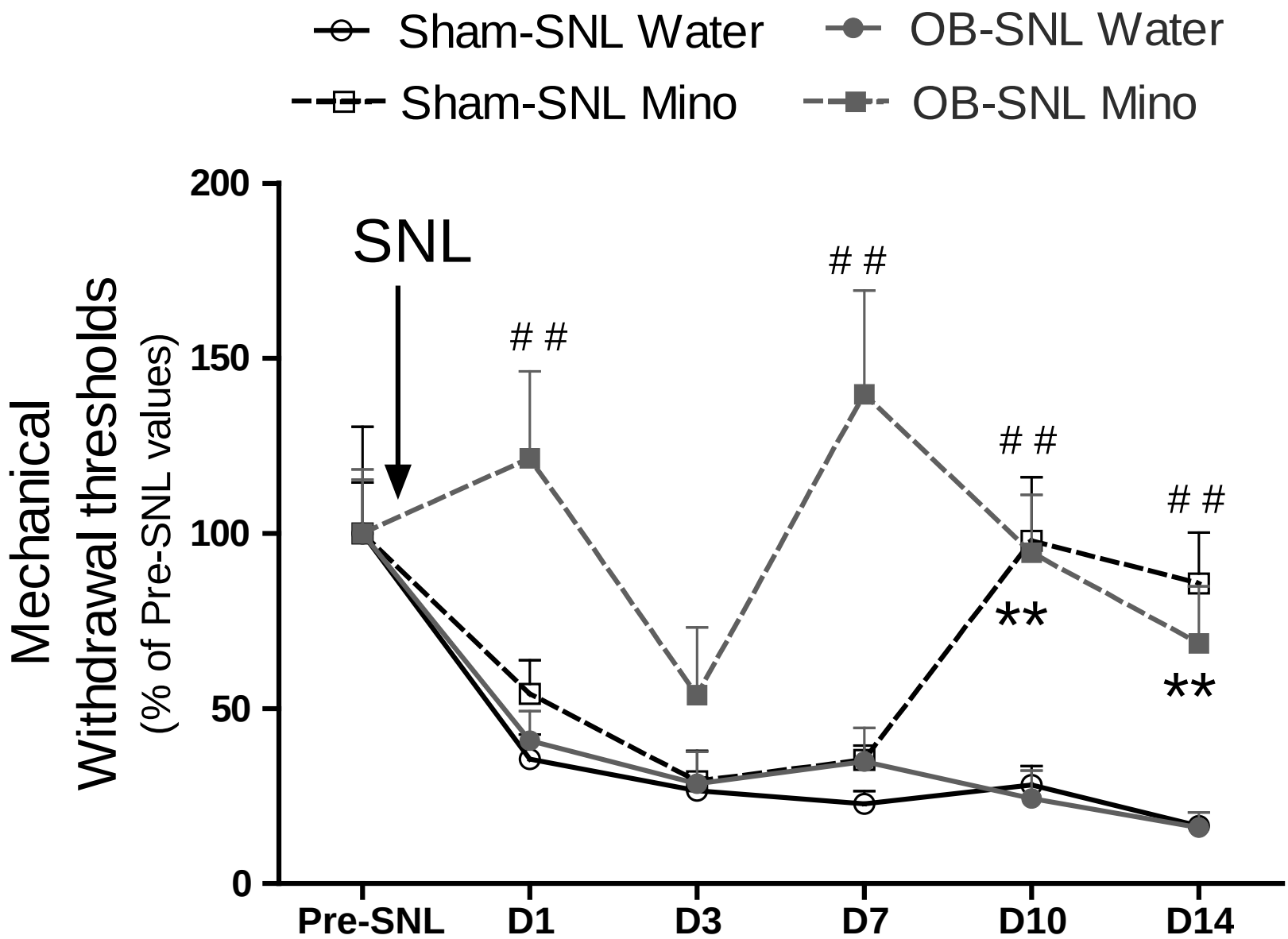
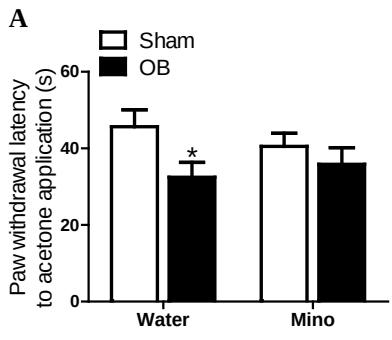
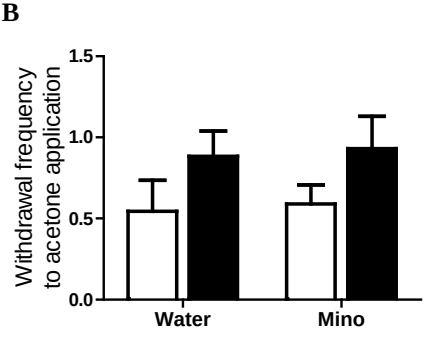

Sham-SNL Water
OB-SNL Water
Sham-SNL Mino
OB-SNL Mino
Mechanical Withdrawal thresholds
(% of Pre-SNL values)
SNL
200
150
100
50
0
# #
# #
# #
# #
**
**
Pre-SNL
D1
D3
D7
D10
D14

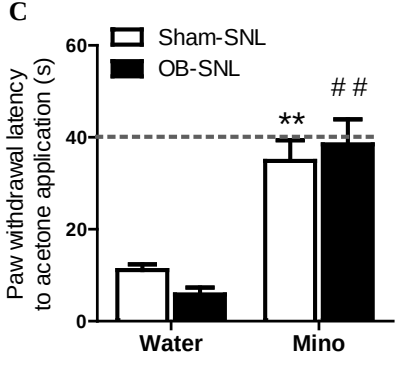
Fig. 3

Pre-SNL





Post-SNL



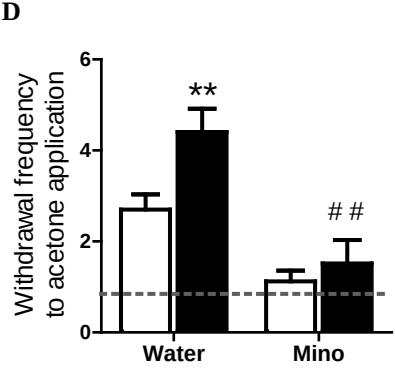
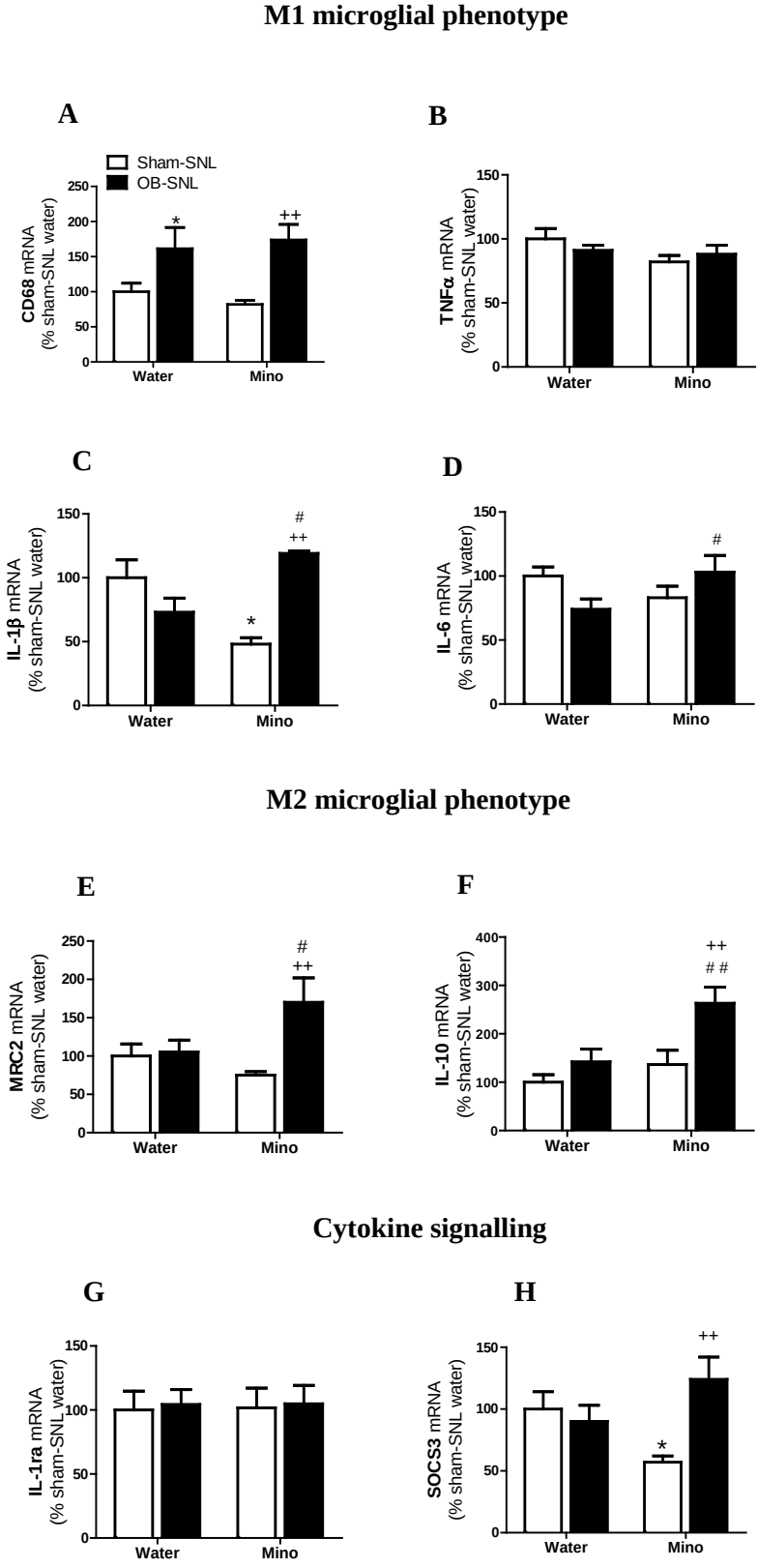


Fig. 5


M1 microglial phenotype
A
Sham-SNL
OB-SNL
CD68 mRNA (% sham-SNL water)
250
200
150
100
50
0
*
++
Water
Mino
B
TNFα mRNA (% sham-SNL water)
150
100
50
0
Water
Mino
C
IL-1β mRNA (% sham-SNL water)
150
100
50
0
*
#
++
Water
Mino
D
IL-6 mRNA (% sham-SNL water)
150
100
50
0
#
Water
Mino
M2 microglial phenotype
E
MRC2 mRNA (% sham-SNL water)
250
200
150
100
50
0
#
++
Water
Mino
F
IL-10 mRNA (% sham-SNL water)
400
300
200
100
0
++
# #
Water
Mino
Cytokine signalling
G
IL-1ra mRNA (% sham-SNL water)
150
100
50
0
Water
Mino
H
SOCS3 mRNA (% sham-SNL water)
150
100
50
0
*
++
Water
Mino