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Oral 2-hydroxyoleic acid inhibits reflex hypersensitivity and open–field-induced anxiety after spared nerve injury

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Conflicts of interest
None declared.

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Abstract

Background: Recently, fatty acids have been shown to modulate sensory function in animal models of neuropathic pain. In this study, the antinociceptive effect of 2-hydroxyoleic acid (2-OHOA) was assessed following spared nerve injury (SNI) with reflex and cerebrally mediated behavioural responses.
Methods: Initial antinociceptive behavioural screening of daily administration of 2-OHOA (400 mg/kg, p.o.) was assessed in Wistar rats by measuring hindlimb reflex hypersensitivity to von Frey and thermal plate stimulation up to 7 days after SNI, while its modulatory effect on lumbar spinal dorsal horn microglia reactivity was assessed with OX-42 immunohistochemistry. *In vitro* the effect of 2-OHOA (120 μM) on cyclooxygenase protein expression (COX-2/COX-1 ratio) in lipopolysaccharide-activated macrophage cells was tested with Western blot analysis. Finally, the effects of 2-OHOA treatment on the place escape aversion paradigm (PEAP) and the open–field-induced anxiety test were tested at 21 days following nerve injury compared with vehicle-treated sham and pregabalin-SNI (30 mg/kg, p.o.) control groups.
Results: Oral 2-OHOA significantly reduced ipsilateral mechanical and thermal hypersensitivity up to 7 days after SNI. Additionally 2-OHOA decreased the COX-2/COX-1 ratio in lipopolysaccharide-activated macrophage cells and OX-42 expression within the ipsilateral lumbar spinal dorsal horn 7 days after SNI. 2-OHOA significantly restored inner-zone exploration in the open-field test compared with the vehicle-treated sham group at 21 days after SNI.
Conclusions: Oral administration of the modified omega 9 fatty acid, 2-OHOA, mediates antinociception and prevents open–field-induced anxiety in the SNI model in Wistar rats, which is mediated by an inhibition of spinal dorsal horn microglia activation.

1. Introduction

The spared nerve injury (SNI) model (Decosterd et al., 2004; Scholz et al., 2008) has been used to screen for pharmacological agents designed to mediate analgesia, particularly as it includes clinically relevant symptoms (Decosterd and Woolf, 2000). SNI elicits microglia cell activation within the spinal dorsal horn and contributes to the development of mechanical and thermal reflex hypersensitivity (Scholz and Woolf, 2007; Scholz et al., 2008), partly mediated by COX-1/2 enzyme-dependent mechanisms (Matsui et al., 2010). Following SNI, constitutive COX-1 expression within spinal dorsal horn microglia is increased (Kanda et al.,

What's already known about this topic?
- Oleic acid (OA) modulates rheumatoid arthritis and orofacial pain.
- 2-hydroxy OA (2-OHOA) mediates hypotension and has anti-cancer properties.

What does this study add?
- Oral administration of 2-OHOA inhibits noxious reflex hypersensitivity, open–field-induced anxiety and microglia reactivity within the dorsal horn following spared nerve injury.

2013), while COX-2 up-regulation has been identified in both human and rat tissues following peripheral nerve injury (Durrenberger et al., 2004, 2006).

Previous studies indicate that the ω-9 monounsaturated fatty acid, oleic acid (OA), modulates rheumatoid arthritis (Kremer et al., 1990) and orofacial pain (Vahidy et al., 2006). Several mechanisms of action related to the control of nociception (Wagner et al., 1998; Rode et al., 2005; Scholz et al., 2008; Brenchat et al., 2012; Kanda et al., 2013; Morgenweck et al., 2013) may represent possible targets for modulation by OA. These include inhibition of gamma-aminobutyric acid (GABA) reuptake (Troeger et al., 1984) and activation of serotonin 5-HT7A receptors (Alberts et al., 2001). However the effect of ω-9 monounsaturated fatty acids on behavioural hypersensitivity mediated by microglia activation induced following peripheral nerve injury is not known (Scholz and Woolf, 2007; Scholz et al., 2008).

Intrathecal administration of OA in combination with albumin inhibits noxious cutaneous hindlimb reflex activity following spinal cord injury (Avila-Martin et al., 2011), which is mediated by an increase in serotonin innervation, reversal of N-methyl-D-aspartate receptor subunit NR1 phosphorylation and inhibition of microglia reactivity (OX-42) within the lumbar dorsal horn (Avila-Martin et al., 2011). Considering that these mechanisms of action have been implicated in the control of nociception (Ultenius et al., 2006; Scholz et al., 2008; Brenchat et al., 2012), the hypothesis of this study was that the administration of OA, or its derivatives, could control noxious reflex hypersensitivity that develops following peripheral nerve injury. OA derivatives (e.g., oleamide and nitro-OA) modulate nociception and anxiety in non-injured rats (Fedorova et al., 2001), COX-2 in microglia cell cultures (Oh et al., 2010) and transient receptor potential channel function within the dorsal root ganglia (Zhang et al., 2014).

The bioactive modified ω-9 fatty acid molecule, 2-hydroxy OA (2-OHOA) mediates hypotension and has anti-cancer properties (Martinez et al., 2005; Alemany et al., 2006). Compared with OA, the modified 2-OHOA undergoes a slower metabolization due to the fact that hydroxylation of the alpha carbon impairs beta-oxidation (Alemany et al., 2004; Vogler et al., 2008). To date, the potential analgesic effect of 2-OHOA has not been studied using outcome measures related to both sensory and affective pain dimensions (Baastrup et al., 2010). Here, we show that oral administration of 2-OHOA inhibits both mechanical and thermal hypersensitivity, normalizes chronic pain anxiety behaviour (Seminowicz et al., 2009) and reduces microglia reactivity within the lumbar spinal dorsal horn following SNI.

2. Methods

2.1 Animals

Following approval by the institutional animal experimentation ethical committee, 10-week-old male Wistar rats (250–300 g; Harlan Laboratories) were maintained in the animal resource unit with food and water provided *ad libitum*. The experiments adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983).

2.2 Experimental design and administered drugs

The analgesic effect of 2-OHOA on both nociceptive and cerebrally mediated behavioural tests in animals with SNI was assessed in 47 rats. The fatty acid 2-OHOA was obtained from Lipopharma (Palma de Mallorca, Spain), dissolved in distilled water and administered by oral gavage (p.o.) at a dose of 400 mg/kg. This dose was chosen according to previous studies, which demonstrated that 2-OHOA was antihypertensive when administered between doses of 100 and 900 mg/kg, p.o., every 12 h produced (Alemany et al., 2006), and a lack of obvious toxic effects based on cytohistological analysis of different organs following administration at 1200 mg/kg, p.o. (Martinez et al., 2005). Pregabalin (Pfizer La Moraleja, Alcobendas, Madrid, Spain) was administered at 30 mg/kg, p.o. in water. Both compounds and the vehicle 0.5 mL, p.o. were administered immediately after SNI and every day thereafter, 2 h before behavioural analysis.

The antinociceptive effect of 2-OHOA was assessed with an initial set of behavioural tests to provide proof of concept, that included measuring nociceptive spinal reflex activity in response to different stimuli. This initial screening assessed SNI animals treated with vehicle or 2-OHOA- ($n = 8$ per

group) up to 7 days after injury. Following initial demonstration of the effect of 2-OHOA on reflex hypersensitivity following SNI, a second comprehensive behavioural study was implemented using sham-controlled behavioural analysis up to 21 days after SNI, to determine sensitivity to low- and high-intensity mechanical stimuli, to phasic application of cold stimuli, as well as cerebrally mediated behavioural tests that included the place escape avoidance paradigm protocol (PEAP) and the open-field anxiety test. These cerebrally mediated tests were incorporated to assess the effect of 2-OHOA on both the affective component of pain and related co-morbidities such as anxiety. In this second study, the animals were distributed among the following four experimental groups: (1) sham surgery ($n = 8$); (2) vehicle-treated SNI ($n = 7$); (3) pregabalin-treated SNI ($n = 8$); and (4) 2–OHOA-treated SNI group ($n = 8$).

2.3 SNI model

The SNI model has been used to study neuropathic pain mechanisms and analgesic efficacy of new pharmacological treatments (Decosterd and Woolf, 2000; Decosterd et al., 2004). This model develops relatively stable behavioural signs of change in nociceptive function, including cold sensitivity, which is relevant for the testing of new analgesic drugs (Decosterd and Woolf, 2000; Erichsen and Blackburn-Munro, 2002; Decosterd et al., 2004). Surgery was performed by inducing anaesthesia with 2.0% isofluorane in 1.0–1.5 L/min medicinal air. Sham SNI surgery was performed by exposing the sciatic nerve at the mid-thigh level, without nerve ligation, followed by suturing the muscle and skin layers together. In the full SNI surgery, the sciatic nerve was exposed as before, but the tibial and peroneal nerves were then ligated with 6-0 suture silk and cut with jewellers scissors, with particular attention made not to damage the sural nerve (Decosterd and Woolf, 2000). The wound was closed by suturing the muscle and skin layers with 4-0 reabsorbable synthetic suture.

Motor activity, monitored routinely 4 h after drug administration using the Rota Rod Test (4600, Ugo Basile Srl, Gemonio, Varese, Italy), was assessed to control for direct effects of the pharmacological agents on lower limb motor activity (Avila-Martin et al., 2011).

2.4 Behavioural testing of nociceptive reflex function

For behavioural analysis of nociceptive reflex function, rats were placed individually in plastic boxes ($16 \times 18 \times 17$ cm) with a wire mesh floor and two equal compartments, supported by an elevated platform that permitted clear observation and access to the plantar surface of the paw for mechanical and cold sensitivity testing. The animals were acclimatized for a period of 15–30 min before reflex testing. In the initial screening, the mechanical antinociceptive effect of the pharmacological agents (proof of concept study) behavioural testing was performed using the application of von Frey filaments (Touch Test Sensory Evaluators 5.07, North Coast Medical and Rehabilitation Products, Morgan Hill, CA, USA). Evoked reflex activity was considered when at least three hindlimb withdrawal responses were obtained and defined as defined as threshold. Mechanical hindlimb reflex sensitivity to von Frey stimulation was assessed at both 2 and 4 h following drug administration.

Hindlimb sensitivity to heat or cold stimuli following SNI was tested in rats by placing them on a preheated Peltier controlled metal plate set at 50.0 ± 0.3°C or 0.0 ± 0.3°C, respectively (Teka, Chicago, IL, USA). The time at which the first reflex response was evoked was recorded with a digital stopwatch, with the response being characterized as a movement of the hindlimb ipsilateral to the SNI, or directed behaviour such as licking, orientation or jumping. Three reflex responses were recorded with a minimum interval of 20 min between each test (1 h total testing time). A cut-off latency of 10 s was imposed to avoid tissue damage with exposure to the thermal plate.

The von Frey test was performed by an independent observer at 2 and 4 h after drug administration, followed by the heat and cold plate test, with a 5-min interval between each test.

In the second screening phase of behavioural nociceptive reflex testing, mechanical sensitivity to low-intensity mechanical stimuli was assessed using the Dixon method (Dixon, 1980; Chaplan et al., 1994; King et al., 2009) and in response to a high-intensity pinprick stimulation (Pertin et al., 2007), following application to the lateral plantar surface of the ipsilateral foot, from 3 to 14 days after SNI. Localized and controlled testing of cold sensitivity of the hindlimb ipsilateral to the SNI was performed, to avoid compensatory behaviours from the other limbs, following application of one drop of absolute acetone to the lateral sural nerve territory of the paw. Withdrawal reflex duration was measured with a digital stopwatch (Erichsen and Blackburn-Munro, 2002).

2.5 PEAP and open-field anxiety

The cerebral-mediated behavioural tests were performed once at 21 days after SNI by the same observer (GAM) after a week in a specially acclimatized testing room. The PEAP relies on the measurement of a learning behaviour associated in response to a novel aversive environment (LaBuda and Fuchs, 2000a). The aversive stimulus consisted in applying every 15 s a 15-g von Frey filament to the lateral plantar skin area ipsilateral to the SNI with the animal placed within a test chamber ($70 \times 30 \times 30$ cm) during a 30-min test period, in a white-sided and black-sided chamber with constant and equally distributed lighting (LaBuda and Fuchs, 2000a). The filament was strong enough to initiate a moderate hindlimb withdrawal reflex response to a single application of the 15-g von Frey filament to animals with either sham or SNI during the pilot test (Baastrup et al., 2010). The test was initiated by placing the animal in the middle of the test box whereupon it was permitted to acclimatize for

5 min. Throughout the 30-min test period, the 15-g von Frey filament was applied to the paw ipsilateral (black chamber) or contralateral to the SNI (white chamber), while the animals were allowed to move freely. The time spent either within the white- or black-sided chambers were digitally quantified with an array of infrared detectors within the apparatus (Cibertec SA, Madrid, Spain). The percentage of time spent within the white-sided chamber and the total number of crossings between chambers were analysed as variables of escape/avoidance learning.

Open–field-induced anxiety was assessed in rats placed into a 100 × 100-cm arena made of black Plexiglas, surrounded by a curtain to exclude visual cues outside of the observation area and illuminated within by a 4 lux light source (Cibertec SA). On day 21 after SNI motor behaviour was measured in the arena with a digital camera (Euresys Picolo Liege, Belgium) and movement was digitized within the open-field up to 5 min with commercial software (Ethovision XT 8.0, Noldus, Wageningen, Netherlands). The total time in which the animal spent within the inner 60 × 60 cm was calculated in addition to the total distance covered (Gregoire et al., 2012).

2.6 Assay of 2-OHOA on COX-1 and COX-2 expression *in vitro*

The effect of 2-OHOA on COX-1/2 activity was assessed using macrophage cells (Cayman Chemicals; Ann Arbor, MI, USA). For COX-1 activity, oxidized N,N,N′,N′-tetramethyl-*p*-phenylenediamine was quantified at 590 nm, while COX-2 activity was quantified by the level of prostaglandin H_2 (PGH_2) produced by the reaction. The human monocyte U937 cell line was kindly provided by Dr Amanda Iglesias (Hospital Son Dureta, Palma de Mallorca). These cells were cultured at 37°C in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, penicillin (100 units/mL) and streptomycin (0.1 mg/mL), in 5% CO_2. U937 cells were differentiated using six-well collagen-coated plates at a density of 8×10^5 cells/well in the presence of 100 mM phorbol myristate acetate diluted in dimethyl sulphoxide, 0.1% final concentration) and incubated for 72 h. Differentiated macrophage-like cells were morphologically and molecularly characterized (data not shown). Once differentiated, the culture medium was removed and replaced with fresh medium without inducement medium.

Immunoblotting was used to quantify COX-1 and COX-2 protein expression, in differentiated U937 cells in the presence or absence (control) of 2-OHOA (120 μM) for 1 h. The cells were then washed twice with phosphate-buffered saline (PBS). Cells previously treated with 2-OHOA were then challenged for 6 h with lipopolysaccharide (LPS, 62 ng/mL) plus 2-OHOA (120 μM) in fresh medium, while untreated control cells were challenged with LPS alone (62 ng/mL). Finally, cells were harvested in 200 μL of protein extraction buffer [10 mM Tris-HCl buffer (pH 7.4), 50 mM NaCl, 1 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulphate (SDS), 5 mM iodoacetamide and 1 mM phenylmethanesulfonyl fluoride]. Cell suspensions were sonicated for 10 s at 50 W using a sonicator (Braun Labsonic U, B. Braun Melsungen AG, Melsungen, Germany) and mixed with 10× electrophoresis loading buffer [120 mM Tris-HCl (pH 6.8), 4% SDS, 50% glycerol, 0.1% bromophenol blue, 10% β-mercaptoethanol] and boiled for 5 min. After electrophoresis (9.5% SDS-polyacrylamide gel electrophoresis) and immunoblotting, the membranes (Whatman Protran®, Dassel, Germany) were blocked for 1 h at room temperature in PBS containing 5% non-fat dry milk, 0.5% bovine serum albumin and 0.1% Tween 20 (blocking solution). The membranes were probed overnight at 4°C with one of the following primary antibodies diluted in blocking solution: monoclonal anti-COX-1/anti-COX-2 (1:800; Biotechnology Inc., Santa Cruz, CA, USA), or anti-α-tubulin (1:10,000; Sigma-Aldrich, Madrid, Spain). Finally, the membranes were washed and incubated for 1 h at room temperature in fresh blocking solution containing horseradish peroxidase-linked goat anti-mouse immunoglobulin G (IgG) (1:2000; Amersham Pharmacia, Biotech Inc., Piscataway, NJ, USA). Immunoreactivity was detected by enhanced chemiluminescence (Amersham Pharmacia, Biotech Inc.).

2.7 Effect of 2-OHOA on lumbar spinal dorsal horn OX-42 expression *in vivo*

Seven days after SNI animals were overdosed with a high concentration of sodium pentobarbital, followed by intracardiac perfusion with saline and 4% paraformaldehyde in 0.1 M PB. Spinal tissue was extracted and transferred to 30% sucrose in 0.1 M phosphate buffer and kept at 4°C for at least two days. The lumbar L4–L5 spinal tissue was cut at 30-μm sections in a Leica CM1900 cryostat (Leica Microsistemas S.L.U., Barcelona, Spain), placed onto slides and preserved at –30°C until further analysis. For immunohistochemical analysis, the tissue was preincubated for 1 h in a blocking solution composed of 5% normal goat serum (Fisher Scientific, Madrid, Spain) and 0.2% Triton X-100 prepared in 0.1 M PBS. Tissue was then incubated overnight at 4°C with the monoclonal anti-CD11b antibody for microglia labelling (1:5000, Abcam, Cambridge, UK). After three 10-min washes, an Alexa Fluor 488-linked anti-mouse IgG was incubated for 1 h at 4°C (1:1000; Invitrogen, Alcobendas, Madrid). Finally, sections were mounted with Fluoromount (Sigma-Aldrich, Madrid, Spain) to preserve the fluorescence signal.

Digital images were obtained with a DM5000 B Leica with a digital Leica DFC350 FX camera and were processed with the academic shareware software (NIH Image J 1.38×, National Institutes of Health, Bethesda, MD, USA). Quantification of the OX-42 positive density in the dorsal horn laminae I-V was performed using 20× magnification (with a minimum of three images/tissue section/animal; $n = 5$ rats/group) and measured in μm^2 (Avila-Martin et al., 2011). All analytical procedures were performed with an observer blinded to the specific experimental conditions for each animal.

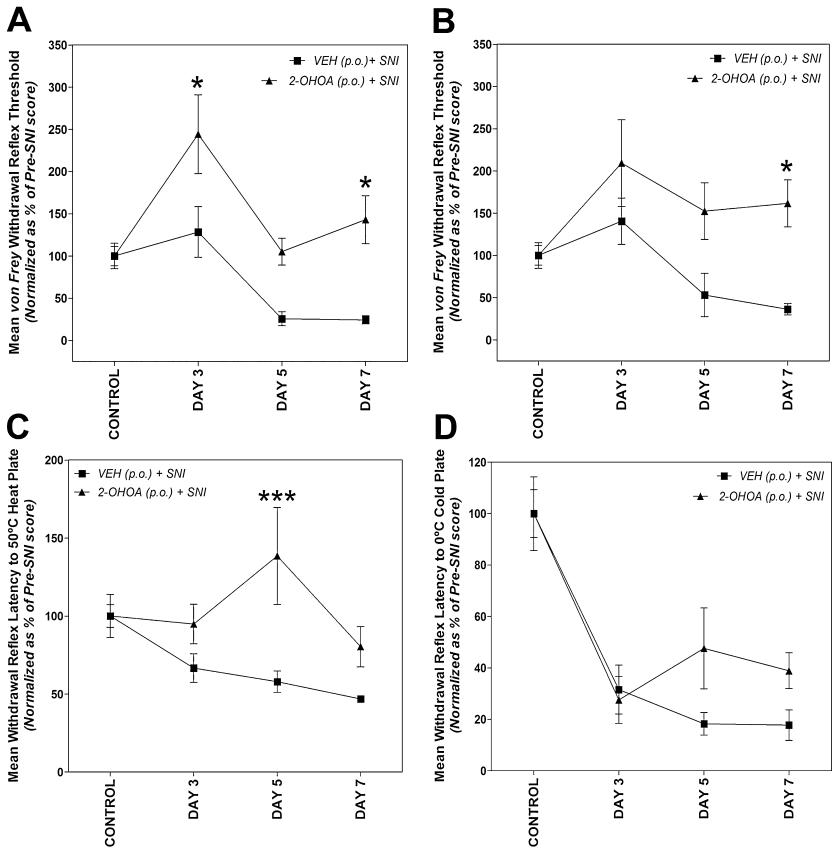


Figure 1 Effect of 2-hydroxy oleic acid (2-OH-OA) treatment (400 mg/kg, p.o.) on hindlimb withdrawal reflex activity measured up to 7 days ipsilateral to the spared nerve injury, compared with vehicle (VEH) administration. Mean von Frey mechanical reflex threshold measured (A) at 2 h or (B) 4 h after treatment. Mean thermal reflex latency in response to (C) 50°C heat plate and (D) 0°C cold plate. Statistical analysis performed with a two-way analysis of variance and Bonferonni test (*$p < 0.05$; ***$p < 0.001$).

2.8 Data analysis and statistical tests

Statistical analysis was performed with Graphpad Prism 5.00 (GraphPad Software, La Jolla, CA, USA). Data were analysed using a one or two-way analysis of variance with a Bonferroni multiple comparison, and the Student's *t*-test according to the study performed (see figure legends).

3. Results

3.1 Initial screening of 2-OHOA for reflex modulation after SNI

Neither the vehicle-treated SNI group (295 ± 5 to 248 ± 35 s) nor the 2–OHOA-treated group (300 ± 0 to 269 ± 16 s) showed a significant reduction in voluntary motor function as tested with the Rota Rod device, or body weight loss, up to 7 days after SNI with 2-OHOA treatment (mean weight increased from 236 to 277 g), suggesting lack of toxicity.

A significant antinociceptive effect of 2-OHOA (400 mg/kg, p.o.) was observed during the 7-day SNI period with von Frey threshold for withdrawal reflex activity 2 h after gavage (Fig. 1A). *Post hoc* analysis revealed higher von Frey thresholds for the hindlimb withdrawal reflex ipsilateral to the SNI following treatment with 2-OHOA at day 3 (244 ± 47% vs. 128 ± 30%, $p < 0.05$) and 7 (143 ± 28% vs. 24 ± 5%, $p < 0.05$) compared with controls, which indicated a general reduction in sensitivity to low-intensity mechanical stimuli following peripheral nerve injury (Fig. 1A). When von Frey thresholds were re-evaluated at 4 h following 2-OHOA treatment, a higher threshold for the withdrawal reflex was also revealed, specifically when compared to vehicle-treated rodents with nerve injury (162 ± 28% vs. 36 ± 7%, $p < 0.05$, 7 days; Fig. 1B), suggesting an antinociceptive effect from 2–4 h after oral administration.

One week treatment with 2-OHOA (400 mg/kg, p.o) administered daily also demonstrated significant antinociceptive activity when withdrawal reflex activity was assessed following heat (Fig. 1C). Reflex hypersensitivity to noxious heat (50°C) stimulation indicated that the 2-OHOA treatment effect was evident at day 5 after SNI (138 ± 31% vs. 58 ± 7%, $p < 0.001$, Fig. 1C). In contrast, no significant modulation of reflex sensitivity to noxious cold stimuli was observed in the 2–OHOA-treated SNI group (Fig. 1D).

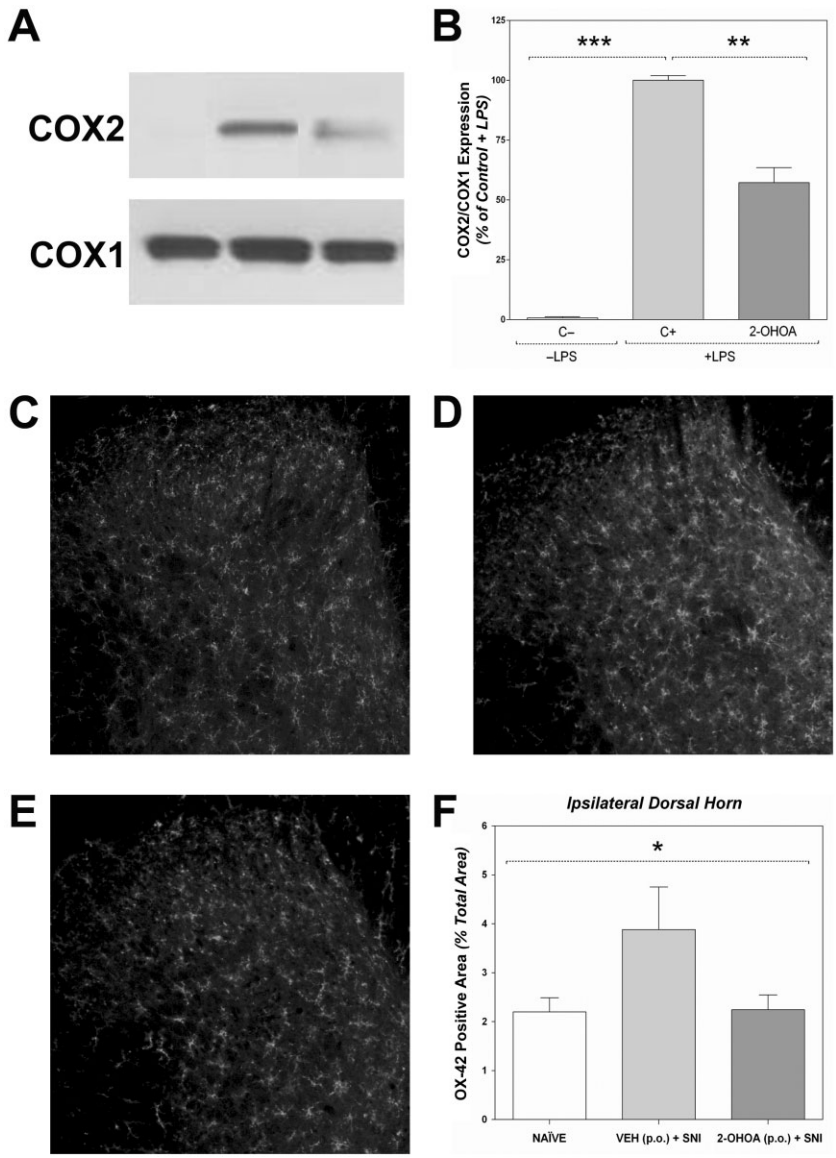


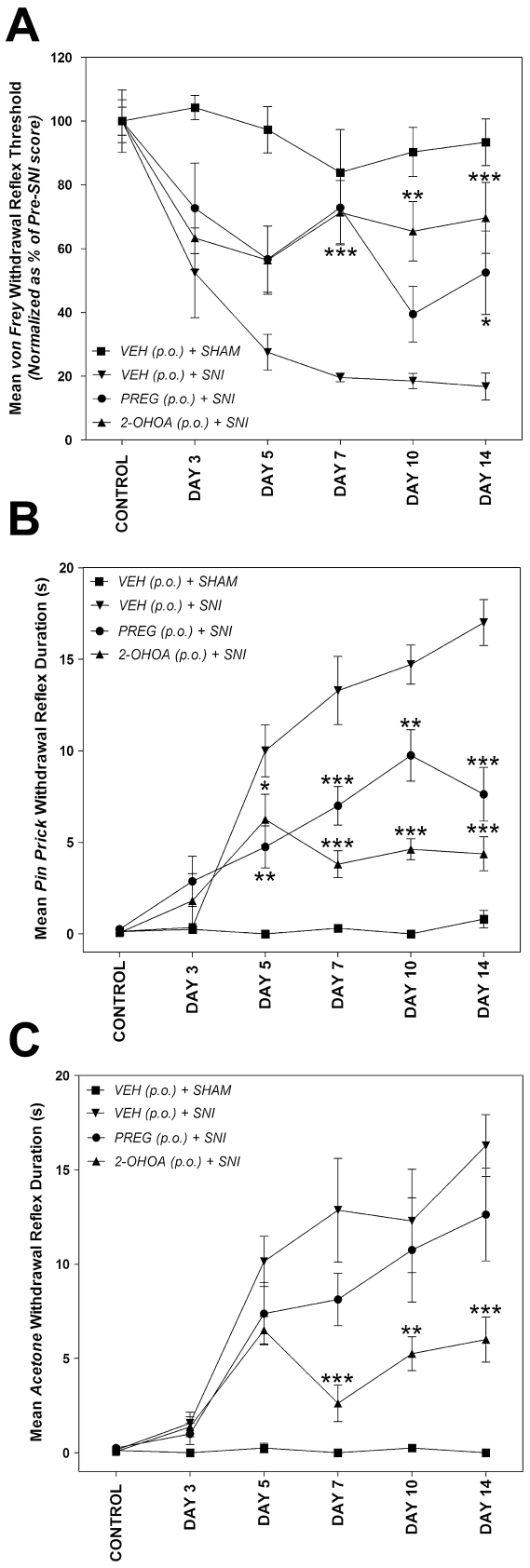
Figure 2 Effect of 2-hydroxyoleic acid (2-OHOA) treatment on LPS-induced macrophage activity *in vitro* (A and B) and on microglia reactivity *in vivo* within the dorsal horn ipsilateral and at 7 days after the spared nerve injury (SNI), compared with vehicle (VEH) administration (C–F). Representative Western blot analysis of cyclooxygenase (COX) 2 expression and COX 1 constitutive expression in cultured macrophage cells (A) before (left column), and 6 h after lipopolysaccharide (LPS) treatment alone (middle column) or with LPS and 2-OHOA (120 μM, right column). Quantification of COX2/COX1 protein expression and the effect of 2-OHOA is presented (B). Representative images (10×) of OX-42 immunostaining in the dorsal lumbar spinal cord from the (C) naïve group, (D) ipsilateral to the SNI in the VEH-treated group and (E) 2–OHOA-treated group (400 mg/kg, p.o.). Quantification of OX-42 expression within the ipsilateral dorsal horn and the effect of 2-OHOA *in vivo* is presented (F). Statistical analysis performed with a one-way analysis of variance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.2 2-OHOA modulation of LPS-activated macrophage COX-2/COX-1 expression

The effect of 2-OHOA treatment on LPS-induced macrophage activity *in vitro* was assessed by COX-1 and COX-2 immunoblotting (Fig. 2A and B). COX-1 expression was not increased in cultured macrophages when compared before or after LPS insult in the presence or absence of 120 μM 2-OHOA (Fig. 2A). However a partial reduction of COX-2 expression was produced following 2-OHOA treatment in the presence of LPS (Fig. 2A). Quantification of the COX-2/COX-1 expression ratio demonstrated an increase from $0.8 \pm 0.5\%$ to $100 \pm 2\%$ ($p < 0.001$) following the 6 h LPS insult (Fig. 2B). Furthermore, treatment with 2-OHOA (120 μM) decreased the COX-2/COX-1 expression ratio in LPS-activated macrophage cells to $57 \pm 6\%$ ($p < 0.01$, Fig. 2B).

3.3 2-OHOA modulation of lumbar dorsal horn microglia reactivity after SNI

A general increase in microglial reactivity was observed in the L4–L5 dorsal horn ipsilateral to the SNI, 7 days after injury, compared to naïve rats (Fig. 2C and D). Animals treated with 2-OHOA showed a decrease in OX-42 (CD11b)-positive area within the dorsal horn (Fig. 2E), when compared with vehicle-treated SNI animals (Fig. 2D). The OX-42 positive area within the L4–L5 dorsal horn represented $2.2 \pm 0.3\%$ of the total



area in naïve animals, compared to 3.9 ± 0.9% of the area in the vehicle-treated SNI group (Fig. 2F). Oral treatment with 2-OHOA (400 mg/kg, 7 days, p.o.) decreased the OX-42 positive area to the basal control level (2.2 ± 0.3%).

3.4 Comprehensive and controlled assessment of 2-OHOA reflex modulation after SNI

Reflex sensitivity was performed following SNI to von Frey, pinprick and acetone stimuli applied specifically to the lateral plantar skin area.

Administration of 2-OHOA (400 mg/kg, p.o.) significantly reduced hyperreflexia evoked in response to von Frey filament stimuli application after SNI. Following SNI, the withdrawal reflex threshold decreased to 17 ± 4% compared to the pre-injury control, while 2-OHOA treatment significantly increased this threshold to 70 ± 11% compared to the vehicle-treated group ($p < 0.001$), with *post hoc* differences identified from day 7 to 14 (Fig. 3A). The control SNI group treated with Pregabalin treatment also demonstrated an increased von Frey withdrawal threshold at day 14 (53 ± 13%, $p < 0.05$, Fig. 3A).

An antinociceptive effect of 2-OHOA on the duration of the withdrawal reflex evoked by pin-prick stimulation following SNI was also demonstrated compared to the vehicle-treated group (Fig. 3B). Following SNI the ipsilateral withdrawal reflex duration to pin-prick stimulation increased in the vehicle-treated group to 17.0 ± 1.3 s at day 14 after injury compared to the vehicle-treated sham-operated group (0.8 ± 0.5 s). Treatment with 2-OHOA reduced the withdrawal reflex duration to 4.4 ± 0.9 s ($p < 0.001$). Similarly at day 14, 2-OHOA maintained its antinociceptive effect from day 5 onwards. Pregabalin treatment was also effective in reducing withdrawal duration to 7.6 ± 1.5 s ($p < 0.001$) with a similar temporal profile as 2-OHOA.

Administration of 2-OHOA in the SNI model also demonstrated an antinociceptive effect for cold stimuli applied as an acetone drop to the ipsilateral lateral plantar pad, which was not observed with Pregabalin treatment (Fig. 3C). Withdrawal reflex duration

Figure 3 Effect of 2-hydroxyoleic acid (2-OHOA) treatment (400 mg/kg, p.o.) on hindlimb withdrawal reflex activity measured up to 14 days ipsilateral to the spared nerve injury (SNI), compared to sham-operated and SNI animals treated with vehicle (VEH) and pregabalin (PREG, 30 mg/kg, p.o.). (A) Mean von Frey mechanical reflex threshold, (B) mean pin-prick reflex duration and (C) mean acetone reflex duration measured up to 21 days after SNI for each experimental group.

increased to 16 ± 2 s in the vehicle-treated SNI group after acetone administration, compared with the vehicle-treated sham group at day 14 (0 ± 0 s). 2-OHOA significantly inhibited withdrawal reflex duration in response to acetone (6 ± 1 s, $p < 0.001$) tested up to day 14. No antinoceptive effect of Pregabalin was observed in response to acetone following SNI.

When voluntary motor function tested with the Rota Rod device was compared with the pre-injury data no significant effect of either vehicle (292 ± 5 to 298 ± 2 s) or 2-OHOA treatment (296 ± 3 to 294 ± 3 s) was observed at 14 days after SNI.

3.5 2-OHOA modulation of PEAP and open-field anxiety after SNI

Testing with the PEAP failed to demonstrate changes in the vehicle-treated group following SNI. No significant difference was observed in the time spent in the white-walled test chamber for the vehicle-treated sham (58 ± 3%) or vehicle-treated SNI group (67 ± 5%) during the test. Furthermore, no significant effect was observed for either 2-OHOA (72 ± 12%) or pregabalin treatment following SNI (70 ± 13%) when compared with either the vehicle-treated sham or vehicle-treated SNI groups on day 21. Finally no difference was observed in the number of crossings between the two chambers in any of the groups.

In contrast, the open-field test revealed a significant reduction in evoked anxiety in the 2–OHOA-treated SNI group (400 mg/kg, Fig. 4) at 21 days post injury. Animals with SNI treated with pregabalin revealed a tendency to explore the inner zone of the open field (Fig. 4C), while an example of the motor activity exhibited by a rat with SNI treated with 2-OHOA showed complete exploration of the inner zone on day 21 (Fig. 4D). Quantification of the motor activity during the 5-min test period revealed a reduction of the time spent in the inner zone for the vehicle-treated SNI group (7 ± 1%) compared to vehicle-treated animals with SNI (15 ± 1%, $p < 0.001$, Fig. 4E). While pregabalin demonstrated a tendency for an increase in inner-zone exploration (11 ± 2%) compared to the sham-operated control group (15 ± 1%), the 2–OHOA-treated group revealed significant increase in exploration time within this area (14 ± 2%, $p < 0.05$), suggesting a reduction in open–field-induced anxiety behaviour after SNI (Fig. 4E). Importantly, no significant difference was observed between the experimental groups during the 5-min test regarding the total distance travelled (Fig. 4F).

4. Discussion

Oral administration of 2-OHOA demonstrates potential analgesic properties as tested in the SNI model, characterised by a reduction of reflex hypersensitivity to both mechanical and cold stimuli without loss of voluntary motor function. In addition, microglia reactivity within the dorsal horn ipsilateral to the SNI was reduced following 2-OHOA treatment, suggesting a mechanism for the modulation of central neuroinflammation and hyperreflexia. Importantly, 2-OHOA reduced behavioural anxiety associated with SNI, suggesting that it may modulate clinically relevant comorbidities (Breivik et al., 2006).

4.1 2-OHOA modulates stimulus evoked hyperreflexia after SNI

Low-dose morphine, mexiletine and methotrexate decreased hypersensitivity to mechanical and cold stimuli after SNI, while gabapentin only reduced sensitivity to von Frey filaments (Erichsen and Blackburn-Munro, 2002; Scholz et al., 2008). During chronic SNI, morphine, gabapentin and carbamazepine prevent the development of stimulus-induced progressive tactile hypersensitivity (Decosterd et al., 2004), but pregabalin only demonstrated an effect against hypersensitivity to von Frey filament stimulation. Previous studies detected an equivocal effect of pregabalin in peripheral nerve injury models (Gustafsson and Sandin, 2009; Yang et al., 2014). Furthermore, pharmacological modulation of behavioural hypersensitivity to cold and low-intensity punctate mechanical stimuli is challenging with phramacological agents such as gabapentin, oxycodone and amitryptiline (Pradhan et al., 2010). Therefore, the modulation of reflex sensitivity to von Frey, pin-prick or acetone stimuli with 2-OHOA is notable in this study.

Several mechanisms for the modulation of cold sensitivity following peripheral neuropathy could be activated by 2-OHOA. Infiltration of the dorsal root ganglia by macrophage and T-cells (Schmid et al., 2013) and the action of cytokines such as interleukin-6 (IL-6) on increasing cold sensitivity following chronic constriction injury (Vissers et al., 2005), suggest that the inhibition of microglia activation with 2-OHOA treatment would modulate senstivity to cold after peripheral neuropathies. The activation of the peroxisome proliferator activated receptor (PPAR)-γ (Churi et al., 2008) and the serotoninergic system (Rahman et al., 2006) also modulate this sensory modality. These mechanisms, previously shown to be modulated by the application

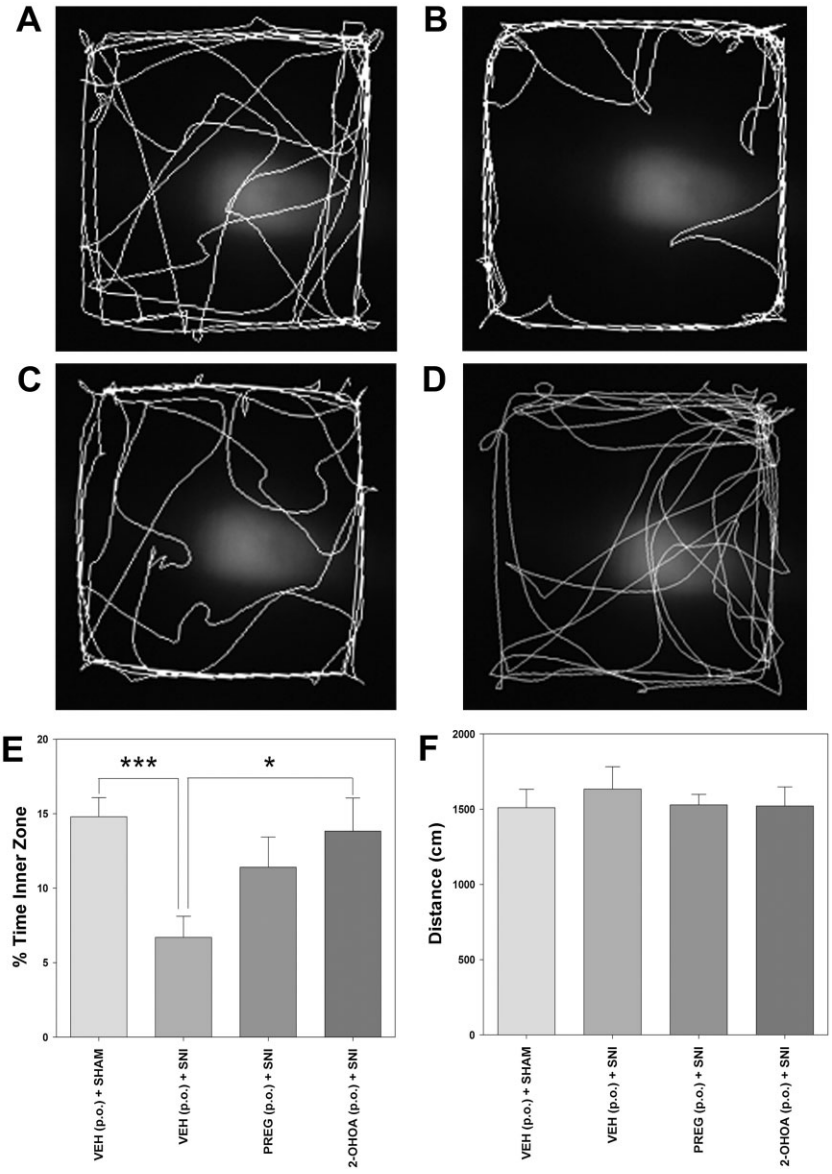


Figure 4 Effect of 2-hydroxyoleic acid (2-OHOA) treatment (400 mg/kg, p.o.) on the open-field anxiety test measured at 21 days ipsilateral to the spared nerve injury (SNI), compared with sham-operated and SNI animals treated with vehicle (VEH) and Pregabalin (PREG, 30 mg/kg, p.o.). Representative open-field trajectories for the (A) sham-operated group treated with VEH (p.o.), (B) SNI group treated with VEH (p.o.), (C) SNI group treated with PREG (p.o.) and (D) SNI group treated with 2-OHOA (p.o.). Analysis of the open-field anxiety test measured (E) as percentage time spent in the inner zone, and controlled (F) for the total distance travelled for each experimental group. Statistical analysis performed with a one-way analysis of variance and *t*-test (*$p < 0.05$; ***$p < 0.001$).

of OA and albumin following spinal cord injury (Avila-Martin et al., 2011; Fandel et al., 2013), could also be activated by the OA analogue 2-OHOA following SNI. In fact, nitro-OA, has been shown to activate transient receptor potential cation channel, subfamily A, member 1 (TRPA1) channel in dorsal root ganglia cells (Zhang et al., 2014), further establishing a potential mechanism of action for inhibition of cold sensitivity with systemic 2-OHOA.

4.2 2-OHOA, inhibition of microglia reactivity and related mechanisms

2-OHOA mediated an inhibition of microglial cell reactivity within the dorsal horn after SNI. In addition, it reduced the COX-2/COX-1 ratio measured in LPS-activated macrophages. Microglia have been implicated in increased excitability of nociceptive dorsal horn neurons and behaviours (Milligan and Watkins, 2009; Aldskogius and Kozlova, 2013; Ji et al., 2013) and are triggered by macrophage colony-stimulating factor, IL-6 and the neuronal chemokine fractalkine (Zhuang et al., 2007; Lee et al., 2010). Oleamide has also been shown to inhibit induction of proinflammatory mediators [e.g., nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)] in microglial cells (Oh et al., 2010). Therefore the effect of 2-OHOA is likely mediated in part by COX-1 and COX-2 dependent processes associated with microglia activation (Matsui et al., 2010).

Other mechanisms related to microglia activation may also be modulated by 2-OHOA. OA and its derivatives activate PPARs (Guzman et al., 2004), while the activation of the PPAR-γ subtype leads to a reduction in glia activation and behavioural hypersensitivity following SNI (Morgenweck et al., 2013). OA is also superior when compared to polyunsaturated fatty acids in reducing lipid peroxidation (Hart et al., 1991) and for the inhibition of glial superoxide radical production (Chan et al., 1988). OA also inhibits gap junction permeability in astrocytes (Lavado et al., 1997) and 2-OHOA could reduce the impact of glia activation on the maintenance of behavioural hypersensitivity after SNI (Wang et al., 2014). Microglia cells are also a major constitutive source of leukotriene B4 *in vitro* (Matsuo et al., 1995), while *in vivo* it mediates mechanical hypersensitivity after SNI (Okubo et al., 2010). Eicosatrienoic acid, an OA derivative, is also a known inhibitor of leukotriene B4 synthesis (James et al., 1993). Taken together these studies suggest that 2-OHOA may modulate behavioural hypersensitivity following SNI through mechanisms related to inhibition of microglia activation within the dorsal horn.

4.3 2-OHOA and inhibition of pain-related anxiety

Pharmacological modulation of reflex hypersensitivity to stimuli following injury to the peripheral (Decosterd and Woolf, 2000) or central nervous system (Avila-Martin et al., 2011), may not reflect affective processing of noxious input in pain models (Johansen et al., 2001; Baastrup et al., 2010). The development of chronic pain affective comorbidities, such as anxiety, represent an important clinical problem (McCracken et al., 1998; Breivik et al., 2006). The identification of anxiety from 21 days after SNI in this study supports these observations, but contrasts with the later development in other models (Seminowicz et al., 2009), where a change in gene expression within the anterior cingulate and frontal cortex in rats has been shown (Seminowicz et al., 2009; Alvarado et al., 2013). However, the detection of anxiety depends on the adopted model, the time after injury and the testing procedure performed (Gregoire et al., 2012). In this study, reduced inner-zone exploration of the open field after SNI was not explained by locomotor disruption, which has been identified as a confounding factor in another study (Gregoire et al., 2012).

Fatty acids demonstrate anxiolytic effects in uninjured rats (Contreras et al., 2011; Borsonelo et al., 2013), similar to the effect of 2-OHOA following SNI. Pregabalin treatment in this study only revealed a tendency as a potential anxiolytic agent after SNI, supporting other studies (Perez et al., 2010; van Seventer et al., 2010). Definitive evidence for the control of SNI-induced anxiety behaviour by 2-OHOA should be obtained with the administration of standard anxiolytic agents such as midazolam (Braun et al., 2011), with a view to identifying common pain-related mechanisms, possibly mediated within the anterior cingulate (Johansen et al., 2001).

4.4 Limitations and future directions

The potential analgesic effect of 2-OHOA demonstrated in the SNI model was not associated with the affective-motivational dimension of pain tested with the PEAP, which has previously been shown to be sensitive to pharmacological modulation in peripheral (LaBuda and Fuchs, 2000b; Pedersen and Blackburn-Munro, 2006) and central pain models (Baastrup et al., 2010). In healthy rodents, anxiety behaviour is related to avoidance responses (Ho et al., 2002). The explanation why this affective behaviour was not identified in the present study may depend therefore on the specific pain aetiology studied, particularly as the PEAP has been performed successfully in inflammatory peripheral nerve injury (LaBuda and Fuchs, 2000a; Pedersen and Blackburn-Munro, 2006) and spinal nerve ligation models (LaBuda and Fuchs, 2000a).

Although 2-OHOA treatment was demonstrated to reduce COX-2/COX-1 expression in LPS-activated macrophage cell activity *in vitro*, in addition to microglia reactivity within the ipsilateral spinal dorsal horn at 7 days after SNI, further information regarding specific mechanisms that mediate the potential analgesic effect of this modified fatty acid should be obtained. Intrathecal OA and albumin administration leads to a down-regulation of PPAR-α expression around the site of spinal cord injury (Fandel et al., 2013), providing further indirect evidence that OA and its derivatives may modulate nociception following endogenous PPAR-α receptor modulation (Guzman et al., 2004). Several PPAR agonists reduce hyperreflexia following peripheral nerve injury (Fehrenbacher et al., 2009), including the PPAR alpha subtype (LoVerme et al., 2006). Whether the modulation of PPAR receptors with 2-OHOA after SNI is mediated within the peripheral or central nervous system should be addressed (Fehrenbacher et al., 2009).

4.5 Conclusion

The potential analgesic effect of oral 2-OHOA demonstrated in the SNI model was highlighted by the inhi-

bition of mechanical and thermal hyperreflexia and the reduction of anxiety behaviour. Inhibition of microglia reactivity elicited following SNI further supports the use of modified omega 9 fatty acids to control both inflammatory and neuropathic pain. Preclinical studies should now be directed to comprehensively evaluate both peripheral and central mechanisms of action of 2-OHOA as a potential analgesic agent and to identify its potential in the prevention of chronic pain measures in other standardized models.

Author contributions

All authors have discussed the results and commented on the paper. G.A.M. designed, conducted, analysed and wrote the paper. P.V.E. and I.G.A. conducted, analysed and wrote the paper. A.F.D. conducted and analysed the study. X.B. performed the COX *in vitro* experiment and provided scientific advice. J.G.S. analysed the behavioural data. J.T. conceived, wrote and interpreted the paper.

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