The degree of acute descending control of spinal nociception in an area of primary hyperalgesia is dependent on the peripheral domain of afferent input

[1]Drake R.A.R., [2]Hulse, R.P. *[1]Lumb B.M and [1,3]Donaldson L.F

[1]School of Physiology and Pharmacology, University of Bristol, [2]Cancer Biology, University of Nottingham and [3]Arthritis Research UK Pain Centre and School of Life Sciences, University of Nottingham.

Category: Neuroscience – behavioural/systems/cognitive

Key words: Nociception, Noradrenaline, Hairy skin

Total word number:

*address for correspondence:

School of Physiology and Pharmacology, The University of Bristol, Medical Sciences Building, University Walk, Bristol BS7 8EJ.
Tel: +44 117 331 2313
Fax: +44 117 331 2288
Email: B.M.Lumb@bristol.ac.uk

Abstract

Descending controls of spinal nociceptive processing play a critical role in the development of inflammatory hyperalgesia. Acute peripheral nociceptor sensitisation drives spinal sensitisation, and activates spino-supraspinal-spinal loops leading to descending inhibitory and facilitatory controls of spinal neuronal activity, that further modify the extent and degree of the pain state. The afferent inputs from hairy and glabrous skin are distinct with respect to both the profile of primary afferents classes and the degree of their peripheral sensitisation. It is not known whether these differences in afferent input differentially engage descending control systems to different extents or in different ways.

Injection of Complete Freund's adjuvant resulted in inflammation and swelling of hairy hindpaw skin in rats, a transient thermal hyperalgesia lasting <2 hours, and long-lasting primary mechanical hyperalgesia (at least 7d). Much longer lasting thermal hyperalgesia was apparent in glabrous skin (1h up to >72h). In hairy skin, transient hyperalgesia was associated with sensitisation of withdrawal reflexes to thermal activation of either A- and C-nociceptors. The transience of the hyperalgesia was attributable to a rapidly engaged descending inhibitory noradrenergic mechanism, which affected withdrawal responses to both A- and C-nociceptor activation and this could be reversed by intrathecal administration of yohimbine (alpha-2-adrenoceptor antagonist). In glabrous skin, yohimbine had no effect on an equivalent thermal inflammatory hyperalgesia.

We conclude that acute inflammation and peripheral nociceptor sensitisation in hind paw hairy skin, but not glabrous skin, rapidly activates a descending

inhibitory noradrenergic system. This may result from differences in the engagement of descending controls systems following sensitisation of different primary afferent classes that innervate glabrous and hairy skin.

Key Points

- Acute inflammation engages various descending control systems in the brain that alter the resulting inflammatory pain, usually by inhibiting it.
- In this study we looked at the differences in inhibition of acute (up to 3h) inflammatory pain from the smooth (glabrous) and the hairy skin in the rat hind-paw.
- In hairy skin, inflammatory pain is rapidly inhibited by descending systems that release noradrenaline, but not opiates, into the spinal cord.
- In glabrous skin, neither descending noradrenergic nor opioidergic controls affect inflammatory pain.
- These results tell us that the controls on the spinal processing of cutaneous inflammatory pain are different depending on the skin type affected.

Abbreviations

Periaqueductal grey (PAG), locus coeruleus (LC), rostroventral medulla (RVM), Complete Freund's adjuvant (CFA)

Introduction

Tissue damage and subsequent inflammation activates and sensitises peripheral nociceptors. This increases afferent input to central nociceptive circuits and leads to enhanced sensitivity to nociceptive stimuli (hyperalgesia), and sensations of pain to innocuous stimulation (allodynia) (Meyer *et al.*, 2005; Latremoliere & Woolf, 2009). Peripheral nociceptive inputs drive both the sensitisation of spinal nociceptive circuits and the activation of ascending and descending pathways that together modulate transmission, and ultimately perception, of nociceptive information (Mantyh & Hunt, 2004). The final perception of the intensity and quality of pain is determined by the degree of such modulation of information throughout the nociceptive neuraxis (Ossipov *et al.*, 2010).

It is well established that spinal nociceptive processing is modulated by both inhibitory and facilitatory descending pathways that originate supraspinally (Millan, 2002). The midbrain periaqueductal grey (PAG) forms a major component of the descending nociceptive system and relays via brain stem nuclei such as locus coeruleus (LC) and the rostroventral medulla (RVM) (Mantyh, 1983; Bajic & Proudfit, 1999) to exert controls on spinal nociceptive neurons. Descending controls from the PAG include opioidergic (Przewłocki & Przewłocka, 2001;Vasquez & Vanegas, 2000; Kwok *et al.*, 2013) and monoaminergic inhibition (Pertovaara, 2006) as well as prostanergic facilitation (Oliva *et al.*, 2006; Leith *et al.*, 2007; Palazzo *et al.*, 2011). Medullary and brain stem nuclei are major sources of spinally projecting monoaminergic fibres (Westlund *et al* 1983; Yoshimura & Furue, 2006) that are known to exert inhibitory influences, which modulate the development of

acute and persistent pain states (Vanegas & Schaible, 2004; Pertovaara, 2006, 2013; Yoshimura & Furue, 2006). Descending noradrenergic systems exert these inhibitory influences largely through presynaptic inhibition, primarily through alpha-2-adrenoceptor activation (Pertovaara, 2006; Yoshimura & Furue, 2006). Alpha-2-agonists, in particular those with affinity for alpha 2A receptors, are antinociceptive in both animals and humans (Pertovaara, 2006). Descending serotonergic systems exert complex pro- and anti-nociceptive effects at the spinal level, primarily through 5-HT_1 and 5-HT_3 autoreceptors (Yoshimura & Furue, 2006; Dogrul *et al.*, 2009; Jeong *et al.*, 2012). Additionally there are peripheral sources of monoamines, such as sympathetic fibres within the dorsal root ganglia, that are able to impact upon nociception during periods of prolonged inflammation or tissue damage (Pertovaara, 2006). Peripheral nerve stimulation evokes spinal release of monoamines in the presence of ganglionic blocking agents (Tyce & Yaksht, 1981), however, so it is widely accepted that the major sources of spinal noradrenaline and serotonin are projections from medullary and brain stem nuclei (Pertovaara, 2006; Yoshimura & Furue, 2006).

Acute cutaneous inflammation leads to sensitisation of peripheral and central nociceptive neurons resulting in primary and secondary hyperalgesia. Descending controls of the spinal processing of input from the area of primary hyperalgesia is initially facilitatory from, for example PAG/RVM, but are short-lived (up to 3h), and are then overwhelmed by local spinal and supraspinal inhibitory influences (Ren & Dubner, 1996; Guan *et al.*, 2002; Miki *et al.*, 2002; Vanegas & Schaible, 2004).The dampening effect of the inhibitory systems on

spinal dorsal horn activity effectively reduces the extent of inflammatory pain by limiting central sensitisation (Vanegas & Schaible, 2004).

Current understanding of the development of inflammatory hyperalgesia and the influence of descending controls over spinal nociceptive processing is largely derived from animal models using inflammatory insults to the glabrous skin of the hindpaw. There is little information on the contribution of descending controls to the development or maintenance of primary hyperalgesia in hairy skin, which is a potential shortcoming given this is the more prevalent skin type in mammals. Glabrous cutaneous tissue found on the plantar surface of the paw forms a major informative surface of the body providing critical sensory/discriminative information about the environment. This is reflected by a greater density of primary afferents innervating glabrous tissue and its over representation in cortical somatotopic maps (Blake *et al.*, 2002; Provitera *et al.*, 2007; Boada *et al.*, 2013). There are notable differences in the profile of nociceptive afferent fibres that innervate glabrous and hairy cutaneous tissue, for instance, a threefold higher proportion of fast conducting to slowly conducting fibres innervating glabrous cutaneous tissue (Boada *et al.*, 2013) and a large proportion of C-polymodal nociceptors innervating the hairy skin of the rat hind-paw (Lynn & Carpenter, 1982; Leem *et al.*, 1993). Critically, there are differences in the sensitisation of unmyelinated nociceptive afferents innervating the two skin types. Following application of conditioning stimuli or sensitising agents, a population of polymodal C-nociceptors found in glabrous skin fail to sensitise to thermal stimuli but a similar population of polymodal C-nociceptors found in hairy cutaneous tissue do sensitise, with a lowering of activation threshold and/or

increased suprathreshold response (Campbell & Meyer, 1983; Andrew & Greenspan, 1999; Koerber *et al.*, 2010). We hypothesised that during inflammation, the differences in primary afferent input from different skin types might differentially drive descending control systems and hence differentially affect the development of inflammatory hyperalgesia in the two skin types.

Materials and Methods

All experiments were performed in accordance with United Kingdom Animals (Scientific Procedures) Act (1986) and associated guidelines. Animals were housed in standard conditions with food and water provided *ad libitum*. Experiments were performed on a total of 58 male Wistar rats weighing 250-350g. The majority of animals (n=55) received a subcutaneous (s.c.) injection of 50 or 100µg Complete Freund's Adjuvant (CFA) (1mg/ml; Cat No: F5881, Sigma-Aldrich, UK) into either dorsal or ventral surface of the left hind-paw under brief halothane (3% in O_2) anaesthesia. Control animals (n=3) received a s.c. injection of an equivalent volume of vehicle (mineral oil). CFA induces a dose dependent increase in swelling, in both rat and mouse, up to doses of 250µg and 500µg respectively. This CFA dose/volume (50µg/50µl) gives a mild, limited inflammation that is less than the maximal achievable with CFA, and that does not spread to involve the sides or other surface of the hindpaw. Greater swelling and spread of inflammation can be seen with higher doses of CFA (Donaldson *et al.*, 1993; Chillingworth *et al.*, 2006). Although differences in oedema have been previously reported with higher CFA doses (Cook & Moore, 2006), we chose to investigate the effects of equivalent inflammatory stimuli in this study, rather than equivalent oedematous responses.

Nociceptive behavioural testing.

To assess the effect of s.c. CFA injections at different sites on nociceptive behaviour, some animals (n=7) underwent nociceptive testing before, three hours, one, three and seven days after either dorsal or plantar s.c CFA injection. Animals were habituated to the apparatus and experimenter

beginning 3 days prior to the start of the testing. Mechanical hyperalgesia: Serrated laboratory forceps were adapted with strain gauges for the measurement of the force applied across the tips (contact area 5.6mm^2 per tip, total 11.2mm^2, Supp. Figure 1). The output signal was fed through a bridge amplifier and captured for subsequent offline analysis via a CED1401 (Cambridge Electronic Design (CED), Cambridge UK) on a computer running Spike2 software (CED, Cambridge, UK). Grams per volt were calculated following calibration with standardised weights placed at the point of finger grip. The gram force delivered was then divided by the total contact area to give grams.mm^{-2}. The tips were placed across the dorsoventral aspect of the hind paw and ramped pressure was applied until the foot was withdrawn or the animal displayed pain-related behaviours (vocalisation, biting etc.) at which point the force applied was immediately stopped. As the duration and force applied were controlled manually, and cut-off on first appearance of pain-related behaviours, the intensity of this stimulus could be controlled to ensure that it did not result in additional tissue damage. Use of this stimulator did not result in any observable tissue damage in naïve or inflamed animals. The occurrence of withdrawal/response was marked using the input from a foot pedal through the CED1401 into a computer running Spike2 software. Two consecutive recordings were made and mean threshold calculated for each animal at each time point.

To test for thermal hyperalgesia a custom built Peltier heating device (contact area 18mm^2) was used to deliver a ramped thermal stimulus to the hind paw of the rat from an initial contact temperature of 30^{o}C. The maximum temperature was cut off at 55^{o}C to prevent tissue damage. Surface (contact)

temperature was measured with a T-type thermocouple (made in-house) and captured for subsequent offline analysis via a CED1401 on a computer running Spike5 software. The extent of paw oedema was assessed by measuring the thickness across the midline dorsoventral aspect of the hind paw using callipers before, and 1 hour, 1, 3 and 7 days after CFA injection.

Surgical preparation

Surgical preparation for electromyograph recordings was performed under initial halothane anaesthesia (2-3% in O_2) and consisted of; (i) external jugular branch cannulation for anaesthetic maintenance (constant intravenous infusion of alphaxalone (~40mg.kg.h; Alfaxan; Jurox Pty PLC, Australia)), (ii) external carotid artery branch cannulation for blood pressure measurement and (iii) tracheal cannulation for airway maintenance. Body temperature was maintained within physiological limits (37-38^{o}C) by means of a feedback controlled heating blanket and rectal probe. For placement of the intrathecal catheter (Størkson *et al.*, 1996), a longitudinal incision was made along the back midline, starting at the level of the iliac crest and advancing 2-3cm rostrally. A 6cm length of 32G polyurethane catheter (OD: 0.25mm, ID: 0.13mm) with a manufacturer-supplied internal metal stylet for increased rigidity (Cat No: 0041; ReCathCo, USA) was back loaded into a 25G needle (Terumo Medical Corporation) so that the end of the catheter was flush with the needle tip. Animals were positioned with the vertebral column flexed and the catheter-loaded needle was inserted bevel up between L5 and L6 vertebrae until a tail flick indicated penetration of dura. The catheter was then advanced ~3cm rostrally along the intrathecal space to approximately the level of the lumbar enlargement. The needle and stylet were then carefully

removed leaving the catheter in place, which was then fixed in position with cyanoacrylate glue (Superglue). A small (~2cm) length of polyethylene tubing (OD: 6.1mm, ID: 2.8mm) was then attached to the free end of the catheter, by insertion of the catheter end just into the polyethylene tubing, which was then secured with cyanoacrylate, to allow for connection to a Hamilton syringe for drug delivery.

For the measurement of electromyographic (EMG) activity, a custom made bi-polar electrode was made using Teflon coated stainless steel wires (0.075mm diameter; Advent research Materials, UK) that were stripped at both ends and one end inserted into the bicep femoris of the hind-leg using a 25-gauge hypodermic needle. The signal across the electrodes was amplified (x1K, Neurolog NL104 amplifier, A-B configuration; Digitimer, UK), filtered (50Hz-5 KHz, Neurolog NL125) and raw data digitised via the CED1401, and stored for offline analysis using Spike2. Animals were left under a constant level of anaesthesia for a minimum of 1 hour after surgical preparation before further experiments. The level of anaesthesia was maintained by constant infusion such that EMG activity could be detected in response to stimulation without overt paw movement.

Preferential activation of A- and C-heat nociceptors.

A- and C-nociceptor were preferentially activated using a custom-made heat lamp in contact with the dorsal hind paw. A constant voltage was applied to the lamp to provide fast ($7.5\pm1^{o}C.s^{-1}$) or slow rates of heating ($2.5\pm1^{o}C.s^{-1}$), which preferentially activate A-fibre (myelinated, capsaicin-insensitive) and C-fibre (unmyelinated, capsaicin-sensitive) heat nociceptors respectively

(Yeomans & Proudfit, 1996; McMullan *et al.*, 2004; McMullan & Lumb, 2006*a*; Leith *et al.*, 2007). Stimuli were applied with an inter-stimulus interval of at least 8 minutes to prevent tissue damage and sensitisation. The heat ramp apparatus was placed in contact with the foot for a minimum of 60 seconds before the start of the heat stimulation to allow for the adaptation for low threshold mechanoreceptors. A feedback-controlled cut-off was set at 58^{o}C for fast thermal ramps and 55^{o}C for slow thermal ramps to prevent tissue damage. In recordings in which thermal ramps reached the cut-off temperature without the occurrence of measurable EMG activity, threshold was recorded as cut-off + 2^{o}C (Leith *et al.*, 2007). For stimulation of glabrous skin, a slow ramp (2.5±1^{o}C.s^{-1}) contact heat stimulus was applied immediately behind the foot-pad using the same apparatus. As it has not yet been determined whether this stimulus is able to preferentially activate A- or C-nociceptors in glabrous skin no attempt was made to draw conclusions with respects to A- or C-nociceptor activation at this site.

Subsurface heating rate measurement

*T*o determine whether CFA-induced paw oedema affected the subcutaneous heating rates that are required for preferential activation of A- and C-nociceptors, subcutaneous heating rates were directly measured using a T-type thermocouple. Heating rates were recorded before, for three hours and on the seventh day after s.c. CFA injection. Care was taken to implant the thermocouple as close to the dermal/epidermal border as possible as nociceptive terminals are found in epidermis and superficial dermal layers. Two ramps were delivered at each experimental time point.

Drugs

The alpha-2-adrenoreceptor antagonist yohimbine 30µg in 10µl in vehicle (80% saline/20%DMSO), Cat no: 1127 Tocris Biosciences, Bristol, UK) was delivered intrathecally via the implanted catheter 2.5h after CFA (n=9) so that the peak drug effect coincided with the third hour following CFA. The concentration of yohimbine used was based on previous reports of effective intrathecal alpha-2 adrenoceptor blockade (Ossipov *et al.*, 1989; Takano & Yaksh, 1992; Green *et al.*, 1998). At the time of injection the tip of a 100µl Hamilton syringe was inserted in to the PE10 tubing and 10µl of the drug was delivered over several minutes. This was washed through with an equivalent volume of saline. In a separate group of animals (n=6) the non-specific opioid antagonist naloxone ($3mg.kg^{-1}$ in 0.9%saline) (Sigma-Aldrich, UK, Cat no: N7758) was delivered intraperitoneally so that the peak drug effect coincided with the second hour following CFA.

At the end of the experiment the position of the cannula was determined by injecting 20µl of Xylocaine (2%) through the cannula. If pinch-evoked EMG activity was abolished the cannula was deemed to be correctly positioned, if not the data were not included in subsequent analyses (n=1, CFA injected). Additionally, in some experiments the location of the distal tip of the catheter was determined by injection of pontamine Sky Blue (20µl of 5%) and visual confirmation of the location of the catheter tip.

Statistical analyses

Data are displayed as mean + SEM unless otherwise noted in Figure legends. Data were analysed using Graphpad Prism v4/5 using Mann Whitney test (2

group comparisons) or Kruskal-Wallis, one- or two-way ANOVA (3 or more groups) where appropriate and as stated in Figure legends. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Results

*Consequences of CFA induced inflammation of the hairy skin of the rat hind paw***.**

Injection of 50µg CFA into the hind-paw dorsum resulted in paw oedema that was evident after one hour and persisted for 7 days (Figure 1A; baseline: 4.2±0.1mm vs seven days: 6.3±0.2mm, $p<0.001$). CFA also produced a mechanical hyperalgesia to a noxious pinch (Figure 1B; baseline 87±33g.mm^{-2} vs. seven days 39±5g.mm^{-2}, $p<0.05$) but did not induce a thermal hyperalgesia in hairy skin (Figure 1C; baseline 49±1.1^{o}C, three hours 49±2.3^{o}C, twenty-four hours 47±1.3^{o}C and three days 51±1.0^{o}C). An equivalent subcutaneous injection of CFA into the plantar surface of the hind-paw resulted in thermal hyperalgesia that persisted for at least three days (Figure 1D, baseline 47±0.9 ^{o}C vs. three days 42±1.2^{o}C, $p<0.05$).

The ability of the thermal ramp stimuli to preferentially activate A- and C-nociceptors is reliant on achieving subsurface cutaneous heating rates of 2.5^{o}C.s^{-1} (fast) for A-nociceptors and 0.8^{o}C.s^{-1} (slow) for C-nociceptors (Yeomans & Proudfit, 1996; McMullan *et al.*, 2004; McMullan & Lumb, 2006*a*; Leith *et al.*, 2007). As paw oedema could affect the heat transfer through the skin, and thus subcutaneous heating rates, we first determined the effect of acute inflammatory oedema on cutaneous heating rates. Surface heating rates elicited by both fast and slow heating ramps were kept within limits previously shown to elicit correct subsurface heating rates in normal skin (Fast - 7.5 ^{o}C.s^{-1}, Slow - 2.5 ^{o}C.s^{-1}; McMullan *et al.*, 2004) and the corresponding subsurface heating rates were recorded. Subsurface heating

rates to both fast and slow thermal ramps were unchanged over the three hours of acute inflammation (Supp. Figure 2, $p>0.05$). However, at seven days following CFA, subsurface heating rates elicited by slow thermal ramps were significantly faster than pre-CFA values (Suppl. Figure 2B; baseline subsurface rate $0.6 \pm 0.03^{o}C.s^{-1}$ vs. seven day subsurface rate $1.2 \pm 0.1^{o}C.s^{-1}$; $p<0.05$).

Effect of CFA induced inflammation on the spinal processing of thermal nociceptor inputs from hairy and glabrous skin.

We sought to determine the effects of CFA induced inflammation of hairy skin on the spinal processing of A- and C-nociceptor inputs. Withdrawal thresholds to thermal A- and C-nociceptor stimulation were both significantly lower one hour after CFA injection (Figure 2A; baseline A-nociceptors vs. one hour A-nociceptors, $57 \pm 0.8^{o}C$ vs. $45 \pm 0.9^{o}C$, $p<0.001$) (Figure 2B; baseline C-nociceptors vs. one hour C-nociceptors, $53 \pm 0.4^{o}C$ vs. $40 \pm 1.8^{o}C$, $p<0.001$). After 2 hours, withdrawal threshold to C-nociceptor stimulation had returned to slightly above baseline levels (Figure 2B; baseline vs. two hours, $53 \pm 0.4^{o}C$ vs. $54 \pm 2.0^{o}C$) whereas A nociceptor thresholds were still lowered (Fig 2A; baseline vs. two hour, $57 \pm 0.8^{o}C$ vs. $49 \pm 2.0^{o}C$, $p<0.001$). By three hours A and C nociceptor thresholds had returned to baseline values (Figure 2A & B, $p>0.05$). Vehicle injection had no significant effect on A- or C-nociceptor thresholds (Fig 2A & B; $p>0.05$). It should be noted that in three of five animals, at two and three hours following CFA, no EMG activity to C-nociceptor stimulation was evoked before stimulation reached the imposed cut-off temperature. Furthermore, in two additional animals (data not used/shown in further analysis) the cut-off was not imposed and in these

animals we found thresholds for withdrawal to C-nociceptor activation to be greater than 60^{o}C. In contrast thresholds of withdrawal to A-nociceptor activation where always below the imposed temperature cut-off.

Doubling the amount of injected CFA resulted in significantly less sensitisation of the reflex pathway over the three hour period (Figure 2C & D). Compared to the 50µg dose, a 100µg CFA injection caused similar reduction in A-nociceptor withdrawal threshold at one hour, but surprisingly significantly less of a reduction in C-nociceptor threshold at this time (Figure 2D; 50µg at 1h vs. 100µg at 1h, 40 ± 1.8^{o}C vs. 51 ± 1.0^{o}C, $p<0.001$). At two hours, both C- and A-nociceptor withdrawal threshold had return to baseline values and A-nociceptor withdrawal thresholds were significantly higher when compared to threshold values for the 50µg dose at the same time point (Figure 2C; 50µl at two hour vs. 100µl at two hour, 49 ± 2.0^{o}C vs 55 ± 1.4^{o}C, $p<0.05$).

Although CFA is known to produce persistent afferent sensitisation lasting days/weeks (Ren & Dubner, 1999), following dorsal CFA we found thermal nociceptive behaviour to be unchanged at 3 days (Figure 1). Additionally, seven days after CFA injection, withdrawal thresholds to A- and C-nociceptor activation were also unchanged (C-nociceptor naïve vs. C-nociceptor seven day CFA, 53 ± 0.6^{o}C. vs. 55 ± 2.0^{o}C; Mann-Whitney $p>0.05$, n=4; A-nociceptor naïve vs. A-nociceptor seven day CFA, 58 ± 0.5^{o}C vs. 58 ± 0.6^{o}C; Mann-Whitney $p>0.05$, n=4, not shown). In contrast, although the withdrawal threshold did increase slightly towards baseline over three hours, injection of CFA into the glabrous skin of the plantar hind-paw resulted in a significant reduction in withdrawal thresholds to the thermal stimulus for the three hours studied (Figure 3 - $p<0.05$ at all time points). It has been previously shown

that s.c. CFA delivered to the plantar surface of the paw leads to thermal hyperalgesia lasting more than one week (Ren & Dubner, 1999). Differences in sensitisation might be attributable to differences in inflammatory reaction and/or oedema. There was significantly greater swelling in the plantar surface of the hindpaw 3 h after injection of CFA compared to the dorsal surface (Figure 4. 3h dorsal 5.9±0.2mm n=7; plantar 6.9±0.2mm, n=6, $p<0.05$). The degree of swelling of the hindpaw was not significantly different after injection of 100µg CFA, compared with 50µg CFA injected into the dorsal surface (Figure 4, 3h 100µg dorsal 6±0.2mm, n=5).

Effect of spinal alpha-2-adrenoceptor receptor blockade on the processing of thermal nociceptor inputs during hind paw inflammation.

Following inflammation of hairy skin the observation of sensitisation followed by a reversal over minutes/hours suggested that descending inhibitory mechanisms could be affecting spinal processing in acute inflammation. Intrathecal delivery of the alpha-2-adrenoceptor antagonist, yohimbine (30µg in 10µl) (Takano & Yaksh, 1992; Green *et al.*, 1998; Hughes *et al.*, 2013), 2 hours after CFA, when thresholds had returned (C-nociceptors) or were returning to baseline (A-nociceptors), significantly inhibited the reversal of both A- and C-nociceptor withdrawal thresholds (Figure 5A; 3 hour A-nociceptor CFA only vs. 3 hour A-nociceptor CFA+yohimbine, 55 ± 1.3^{o}C vs. 50 ± 0.9^{o}C, $p<0.05$) (Figure 5B; 3 hour C-nociceptor CFA only vs. 3 hour C-nociceptor CFA+yohimbine, 55 ± 1.1^{o}C vs. 47 ± 2.3^{o}C, $p<0.05$). In contrast, yohimbine did not affect the withdrawal to thermal stimulation applied to

inflamed glabrous skin (Figure 3), which was apparent at 1 hour, and maintained for the 3 hours studied (Figure 3; CFA only 3h threshold vs. CFA+yohimbine 3h withdrawal threshold, 47 ± 0.7^{o}C vs. 47 ± 0.4^{o}C). Intrathecal delivery of the same volume of vehicle did not affect withdrawal thresholds in either experiment (n=3 for both experiments, data not shown).

Spinal and supraspinal opioidergic systems are known to have robust inhibitory effects on spinal nociceptive processing during inflammation (Stanfa & Dickenson, 1995; Przewłocki & Przewłocka, 2001). We therefore also determined the contribution of opioidergic systems to the degree of hyperalgesia in acute hind paw CFA inflammation. Systemic delivery of the pan-opioid receptor antagonist naloxone (3mg.kg^{-1} (Stanfa & Dickenson, 1995)) had no effect on either A- or C-nociceptor withdrawal thresholds in hairy skin at either 2 or 3 hours following CFA (Figure 5C + D). It has been previously shown that naloxone has no effect on withdrawal latencies to thermal stimuli at 4 hours following plantar inflammation (Hylden *et al.*, 1991; Tsuruoka & Willis, 1996).

Discussion

Two key observations arise from these data; (i) there is a differential descending noradrenergic inhibitory control on different skin types in acute inflammation, and (ii) there is a greater descending inhibitory control of C- compared to A-nociceptor evoked reflexes from hairy skin.

Subcutaneous injection of CFA is a well characterised and widely used cutaneous inflammatory model, primarily used to study inflammatory hypersensitivity in glabrous (plantar) skin (Iadarola *et al.*, 1988; Ren & Dubner, 1999). The oedema generated in glabrous or hairy skin is not equivalent for the same dose of CFA (data herein, (Cook & Moore, 2006)), but as CFA produces a dose-dependent inflammatory response (Donaldson *et al.*, 1993) that should be equivalent at different sites, inflammation of hairy and glabrous skin might be predicted to induce equivalent primary hyperalgesia. Literature reports that CFA injection results in a robust primary mechanical and, usually, following plantar injection, thermal behavioural hypersensitivity to stimulation from as early as one hour and reportedly lasting for weeks (Iadarola *et al.*, 1988; Ren & Dubner, 1999; Terayama *et al.*, 2002; Soignier *et al.*, 2011). Plantar inflammation also results in mechanical hypersensitivity (data herein, (Ren & Dubner, 1999; Cook & Moore, 2006; Soignier *et al.*, 2011). In contrast, dorsal (hairy) hindpaw inflammation results in no change in thermal reflex withdrawal response, despite thermal sensitisation of single peripheral nociceptors in the same model (Dunham *et al.*, 2008; Koerber *et al.*, 2010). CFA induced inflammation results in robust long-lasting peripheral nociceptor thermal sensitisation, in rats, guinea pigs and primates, (Kocher *et*

al., 1987; Davis *et al.*, 1993; Andrew & Greenspan, 1999; Djouhri & Lawson, 1999; Djouhri *et al.*, 2001; Dunham *et al.*, 2008; Koerber *et al.*, 2010) irrespective of whether hairy or glabrous skin is inflamed, which is inconsistent with the behavioural data. We therefore determined whether the observed lack of thermal behavioural responses in acute inflammation might be attributable to descending inhibitory control of nociceptive processing from inflamed hairy skin sites. The observation that plantar inflammation produces greater swelling, but little obvious descending inhibition, whereas dorsal inflammation produces less swelling but more profound inhibition with increasing inflammatory severity, suggest that it is the overall degree of inflammation, rather than just the oedema, that engages descending control systems. This is consistent with previous findings, where much higher CFA doses produced acute thermal analgesia i.e. less nociception than control, in hairy but not glabrous skin (Cook & Moore, 2006). Differences in inflammatory oedema in different sites may not therefore accurately predict nociceptive responses, as this aspect of inflammation may more accurately reflect local tissue compliance (Reed & Rubin, 2010) rather than nociceptive input.

Processing of noxious inputs is known to be subject to both descending inhibitory and facilitatory controls (Millan, 2002; Vanegas & Schaible, 2004). Primary inflammatory hyperalgesia is widely accepted to be dominated by descending inhibitory controls, which are both rapidly activated and long-lasting. These are hypothesised to protect against spinal hyperexcitabilty following inflammation by limiting mechanisms of central sensitisation (Ren & Dubner, 1996; Vanegas & Schaible, 2004). However, they are not usually reported to completely obliterate the nociceptive sensitivity resulting from the

insult (Tsuruoka & Willis, 1996; Tsuruoka *et al.*, 2003; Vanegas & Schaible, 2004). This is in contrast to our findings where thermal hyperalgesia in hairy skin was more short lived, particularly so when inflammation was more severe (100µg CFA dose).

Descending fibres from areas such as LC and RVM exert profound modulatory influences on spinal nociceptive processing (Fields *et al.*, 1991; Tsuruoka & Willis, 1996; Miki *et al.*, 2002; Millan, 2002; Tsuruoka *et al.*, 2003) through monoaminergic systems (Pertovaara, 2006; Yoshimura & Furue, 2006). Descending monoaminergic-systems, particularly noradrenaline acting through spinal alpha-2 receptors, are reported to modulate the *development* of inflammatory hyperalgesia (Tsuruoka & Willis, 1996; Tsuruoka *et al.*, 2003; Pertovaara, 2006). Our findings confirm this for hairy cutaneous tissue, and additionally indicate that, at acute time points, yohimbine blockade *differentially* modulates thermal inflammatory sensitivity in hairy/glabrous skin.

It has been proposed that the descending noradrenergic systems form a stimulus driven negative feedback circuit that impinges onto spinal nociceptive processing and so regulates the onward flow of ascending nociceptive information (Pertovaara, 2006, 2013). In hairy skin, our finding of a greater inhibition in more severe inflammation would support this. In contrast, in glabrous skin hypersensitivity persists (>4h) (Tsuruoka & Willis, 1996) and at acute time points (<3h) we find little evidence of either monoaminergic or opioidergic inhibition, consistent with previous findings (Hylden *et al.*, 1991; Tsuruoka & Willis, 1996).

Although yohimbine has high affinity for alpha-2 receptors and is commonly used as a specific alpha-2-adrenoceptor antagonist, it has moderate affinity at alpha-1-adrenoceptors, 5-HT_{1A}, 5-HT_{1B} and dopamine D2 receptors (K_i determinations generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, (http://pdsp.med.unc.edu/pdsp.php)). As a result of the latter effects, systemic yohimbine can decrease 5-HT and increase dopamine and noradrenaline levels in rat CNS, probably through blockade of autoreceptors (Paalzow & Paalzow, 1983; Millan *et al.*, 2000). In inflammation, spinal 5-HT_{1A} receptors both enhance (Zhang *et al.*, 2001; Wang *et al.*, 2003) and inhibit (Liu *et al.*, 2002; Yu *et al.*, 2011; Horiguchi *et al.*, 2013) acute inflammatory nociception; known descending 5-HT3 receptor-mediated facilitatory pathways do not seem to contribute to inflammatory nociception (Asante & Dickenson, 2010). There are no published data on contributions of spinal D2 receptors to acute inflammatory nociception. Although this concentration of yohimbine administered intrathecally has been widely interpreted as exerting actions primarily at alpha-2 adrenoceptors (Ossipov *et al.*, 1989; Takano & Yaksh, 1992; Green *et al.*, 1998; De Felice *et al.*, 2011; Little *et al.*, 2012; Hughes *et al.*, 2013), we cannot exclude an additional contribution of modulation of alpha-1-adreno or 5-HT receptors at the spinal level to the observed effects of yohimbine. Actions of yohimbine on 5-HT_{1A} receptors would be consistent with a concurrent anti-nociceptive effect of 5-HT in this model (Liu *et al.*, 2002; Yu *et al.*, 2011; Horiguchi *et al.*, 2013).

The nocifensive flexor reflex is thought to provide an objective measure of noxious threshold and is the most commonly used in pain studies in both

humans and animals (Wiesenfeld-Hallin, 1995; Sandrini *et al.*, 2005). In animals the nocifensive flexor reflex typically involves recording electromyographic activity from the biceps femoris evoked by stimulation of the hind-paw (Wiesenfeld-Hallin, 1995), alpha motor neurons serving the biceps femoris have receptive fields on both the dorsal and plantar surface (Cook & Woolf, 1985), thus the effects of drugs on reflexes evoked from both paw surfaces can be directly compared and contrasted. Thus, whilst the musculotopic organisation of reflexes may have meant that recording from different muscular sites may have yielded slightly different results (Schouenborg *et al.*, 1994; Harris & Clarke, 2002), these may not have been directly comparable for stimulation of different sites on the hindpaw. In addition, as alpha-2-adrenoceptor agonists are analgesic (Pertovaara, 2006) and presumably inhibit the sensory afferent arm of the withdrawal reflex, it could be hypothesised that the result of yohimbine blockade would affect, to a greater or lesser extent, all muscle groups involved in noxious withdrawal reflexes. This is supported by the behavioural data.

A differential descending inhibitory control on glabrous versus hairy skin may relate to the functional importance of the footpad/glabrous skin. The plantar surface forms the main exploratory hindpaw surface in rodents, providing important sensory/discriminative information about the environment (Boada *et al.*, 2013). Pain provides a critical protective mechanism in which nociceptive sensitisation, including increased reflex sensitivity, serves to protect injured areas that might regularly come into contact with physical stimuli (Schouenborg *et al.*, 1994) and it has been shown that load bearing parts of the paw have special significance in generating reflex sensitisation (Clarke &

Harris, 2004). However, effective locomotion is essential to survival, being central to feeding and escape behaviours, and therefore the positive impact of nociceptive reflex hypersensitivity and its protective function must be traded off against its negative impact on locomotion. Thus peripheral domains that rarely come into contact with nociceptive stimuli (dorsal hind-paw) may have a reduced capacity to produce reflex sensitisation following inflammation.

The differences in descending inhibition of spinal processing of afferent input from different peripheral domains may also represent a difference in both innervation and the effects of inflammation on innervating neuronal properties. The inflammatory sensitisation of unmyelinated afferents differs in glabrous versus hairy skin in both rat and primate (Campbell & Meyer, 1983; Kocher *et al.*, 1987; Andrew & Greenspan, 1999; Dunham *et al.*, 2008), in that peripheral thermal sensitisation occurs in C-nociceptors innervating hairy skin (Campbell & Meyer, 1983; Davis *et al.*, 1993; Dunham *et al.*, 2008; Koerber *et al.*, 2010), but not in glabrous skin (Meyer & Campbell, 1981; Campbell & Meyer, 1983; Andrew & Greenspan, 1999; Du *et al.*, 2006), where C-nociceptors may become desensitised instead (Campbell & Meyer, 1983; Andrew & Greenspan, 1999). C-nociceptors play critical roles in driving mechanisms of central sensitisation and of descending controls from supraspinal sites (Woolf & Wall, 1986; Sivilotti *et al.*, 1993; Suzuki *et al.*, 2002; Ikeda *et al.*, 2003; Mantyh & Hunt, 2004; You *et al.*, 2010). Sensitisation of C-nociceptors in inflamed hairy skin would drive ascending, and therefore also descending systems to a greater extent than in glabrous skin, where C-nociceptors do not readily sensitise, leading to the differences in inhibition observed. This is supported by the enhanced inhibition seen in a more severe

inflammation where nociceptors would be sensitised more rapidly and afferent barrage would be greater (Figure 3 A & B).

Previous findings from our laboratory indicate that descending inhibitory controls, arising from the PAG preferentially target C-nociceptive inputs to spinal nociceptive networks (McMullan & Lumb, 2006*b*). Here, during acute inflammation of hairy skin, we observe a fast acting and potent inhibition of C-nociceptor evoked withdrawals; at two and three hours following CFA the withdrawal thresholds to C-nociceptor stimulation were greater than baseline values and in 3/5 animals we observed no evoked EMG activity before the cut-off temperature was reached. Additionally, in the two cases where the cut-off was removed, thresholds for EMG activity were greater than 60^{o}C (data not shown). In contrast, at two hours following CFA, thresholds for A-nociceptor evoked withdrawals were still significantly lower than baseline values and, at three hours following CFA, all 5 animals responded to the thermal stimuli before the cut-off temperature was reached. It is likely that the effect of yohimbine on C- versus A-nociceptor evoked withdrawals failed to reach significance (data not shown) as a result of the inherent underestimation of the full effect of yohimbine on C-nociceptor withdrawal thresholds due to the imposed temperature cut-off (55^{o}C). Descending inhibitory systems have been shown to preferentially target thermal spinal nociceptive processing in both normal and inflamed rats (Kauppila *et al.*, 1998; Howorth *et al.*, 2009), consistent with our observations. Additionally, as noxious mechanical stimuli are thought to be conveyed by A-fibre nociceptors (Ziegler *et al.*, 1999; Magerl *et al.*, 2001) an alternative interpretation of these

findings is that there as a preferential inhibition of spinal nociceptive processing of C- versus A-nociceptor inputs.

There may be a possible evolutionary advantage of greater inhibitory control on C- compared to A-nociceptor-evoked reflexes which could relate to the different functions of these afferents. In humans, rapidly conducting A-nociceptors convey sharp, well localised painful sensations (Magerl *et al.*, 2001), information which is encoded with a high fidelity by spinal neurons (rat) (McMullan & Lumb, 2006*a*). In contrast, slowly conducting C-nociceptors convey dull and diffuse pain (Ziegler *et al.*, 1999; Magerl *et al.*, 2001) encoded with low fidelity by spinal neurons in rats (McMullan & Lumb, 2006*a*). Thus A-nociceptors are hypothesised to convey more detailed information relating to the protective function of pain, i.e. localisation and magnitude assessment. A greater acute inhibition of C-nociceptor inputs could serve to filter extraneous C-nociceptive information and limit saturation in the reflex pathway (Tsuruoka *et al.*, 2012) while concurrently preserving A-nociceptive information which is essential to the protective function of reflex withdrawals. Thus differential inhibitory control of inputs from body areas with different functions, and from different nociceptor types would be hypothesised to limit central sensitisation and protect against spinal hyperexcitabilty following inflammation, whilst maintaining adequate locomotor and protective nociceptive function to enable healing/survival.

Competing interests:

The authors have no competing interests to declare.

Author's contributions:

Experiments were performed in laboratories in the School of Physiology and Pharmacology, University of Bristol. RARD, BML & LFD contributed to experimental design, and data analysis and interpretation, RPH designed and built the mechanical stimulator, RARD contributed data collection, RARD, BML & LFD wrote the manuscript, and all authors approved final manuscript submission.

Funding

This work was funded by the Medical Research Council (DTP studentship supporting RARD, Project grant G0801381 (BML)) and a BBSRC/MRC/BPS Strategic Skills award (LFD & BML).

Acknowledgements:

We would like to thank Prof. Bruce Matthews for assistance with the design and build of the mechanical pincher, and additionally Dr. Louise Hickey and Mr Sam Hughes for their helpful discussions and input on technical issues.

Supporting information

The online supporting information gives further detail on the mechanical stimulation device, and the validation of the heat ramp stimulations in inflamed skin.

References:

Andrew D & Greenspan J (1999). Mechanical and heat sensitization of cutaneous nociceptors after peripheral inflammation in the rat. *J Neurophysiol* **82,** 2649–2656.

Asante CO & Dickenson AH (2010). Descending serotonergic facilitation mediated by spinal 5-HT3 receptors engages spinal rapamycin-sensitive pathways in the rat. *Neurosci Lett* **484,** 108–112.

Bajic D & Proudfit HK (1999). Projections of neurons in the periaqueductal gray to pontine and medullary catecholamine cell groups involved in the modulation of nociception. *J Comp Neurol* **405,** 359–379.

Blake DT, Byl NN & Merzenich MM (2002). Representation of the hand in the cerebral cortex. *Behav Brain Res* **135,** 179–184.

Boada MD, Houle TT, Eisenach JC & Ririe DG (2013). Differing neurophysiologic mechanosensory input from glabrous and hairy skin in juvenile rats differing neurophysiologic mechanosensory input from glabrous and hairy skin in juvenile rats. *J Neurophysiol* **104,** 3568–3575.

Campbell JN & Meyer RA (1983). Sensitization of unmyelinated nociceptive afferents in monkey varies with skin type. *J Neurophysiol* **49,** 98–110.

Chillingworth NL, Morham SG, Donaldson LF (2006). Sex differences in inflammation and inflammatory pain in cyclooxygenase-deficient mice. *Am J Physiol Regul Integr Comp Physiol* **291,** 327–334.

Clarke RW & Harris J (2004). The organization of motor responses to noxious stimuli. *Brain Res Brain Res Rev* **46,** 163–172.

Cook AJ & Woolf CJ (1985). Cutaneous receptive field and morphological properties of hamstring flexor α-motorneurones in the rat. *J Physiol* **364,** 249–263.

Cook CD & Moore KI (2006). Effects of sex, hindpaw injection site and stimulus modality on nociceptive sensitivity in arthritic rats. *Physiol Behav* **87,** 552–562.

Davis KD, Meyer RA & Campbell JN (1993). Chemosensitivity and sensitization of nociceptive afferents that innervate the hairy skin of monkey. *J Neurophysiol* **69,** 1071–1081.

Djouhri L, Dawbarn D, Robertson A, Newton R & Lawson SN (2001). Time course and nerve growth factor dependence of inflammation-induced alterations in electrophysiological membrane properties in nociceptive primary afferent neurons. *J Neurosci* **21,** 8722–8733.

Djouhri L & Lawson SN (1999). Changes in somatic action potential shape in guinea-pig nociceptive primary afferent neurones during inflammation in vivo. *J Physiol* **520,** 565–576.

Dogrul A, Ossipov MH & Porreca F (2009). Differential mediation of descending pain facilitation and inhibition by spinal 5HT-3 and 5HT-7 receptors. *Brain Res* **1280,** 52–59.

Donaldson LF, Seckl JR & McQueen DS (1993). A discrete adjuvant-induced monoarthritis in the rat: effects of adjuvant dose. *J Neurosci Methods* **49,** 5–10.

Du J, Zhou S & Carlton SM (2006). Kainate-induced excitation and sensitization of nociceptors in normal and inflamed rat glabrous skin. *Neuroscience* **137,** 999–1013.

Dunham JP, Kelly S & Donaldson LF (2008). Inflammation reduces mechanical thresholds in a population of transient receptor potential channel A1-expressing nociceptors in the rat. *Eur J Neurosci* **27,** 3151–3160.

Fields HL, Heinricher MM & Mason P (1991). Neurotransmitters in nociceptive modulatory circuits. *Annu Rev Neurosci* **14,** 219–245.

Green GM, Lyons L & Dickenson AH (1998). Alpha2-adrenoceptor antagonists enhance responses of dorsal horn neurones to formalin induced inflammation. *Eur J Pharmacol* **347,** 201–204.

Guan Y, Terayama R, Dubner R & Ren K (2002). Plasticity in excitatory amino acid receptor-mediated descending pain modulation after inflammation. *J Pharmacol Exp Ther* **300,** 513–520.

Harris J & Clarke RW (2002). Organisation of sensitisation of hind limb withdrawal reflexes from acute noxious stimuli in the rabbit. *J Physiol* **546,** 251–265.

Horiguchi N, Ago Y, Hasebe S, Higashino K, Asada K, Kita Y, Takuma K & Matsuda T (2013). Isolation rearing reduces mechanical allodynia in a mouse model of chronic inflammatory pain. *Pharmacol Biochem Behav* **113,** 46–52.

Howorth PW, Thornton SR, O'Brien V, Smith WD, Nikiforova N, Teschemacher AG & Pickering AE (2009). Retrograde viral vector-mediated inhibition of pontospinal noradrenergic neurons causes hyperalgesia in rats. *J Neurosci* **29,** 12855–12864.

Hughes SW, Hickey L, Hulse RP, Lumb BM & Pickering AE (2013). Endogenous analgesic action of the pontospinal noradrenergic system spatially restricts and

temporally delays the progression of neuropathic pain following tibial nerve injury. *Pain* **154,** 1680–1690.

Hylden J, Thomas D, Iadarola M, Nahin R & Dubner R (1991). Spinal opioid analgesic effects are enhanced in a model of unilateral inflammation/hyperalgesia: possible involvement of noradrenergic mechanisms. *Eur J Pharmacol* **194,** 135–143.

Iadarola M, Brady L, Draisci G & Dubner R (1988). Enhancement of dynorphin gene expression in spinal cord following experimental inflammation: stimulus specificity, behavioral parameters and opioid receptor binding. *Pain* **35,** 313–326.

Ikeda H, Heinke B, Ruscheweyh R & Sandkühler J (2003). Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. *Science* **299,** 1237–1240.

Jeong H-J, Mitchell V a & Vaughan CW (2012). Role of 5-HT1 receptor subtypes in the modulation of pain and synaptic transmission in rat spinal superficial dorsal horn. *Br J Pharmacol* **165,** 1956–1965.

Kauppila T, Kontinen VK & Pertovaara A (1998). Influence of spinalization on spinal withdrawal reflex responses varies depending on the submodality of the test stimulus and the experimental pathophysiological condition in the rat. *Brain Res* **797,** 234–242.

Kocher L, Anton F, Reeh P & Handwerker H (1987). The effect of carrageenan-induced inflammation on the sensitivity of unmyelinated skin nociceptors in the rat. *Pain* **29,** 363–373.

Koerber HR, McIlwrath SL, Lawson JJ, Malin SA, Anderson CE, Jankowski MP & Davis BM (2010). Cutaneous C-polymodal fibers lacking TRPV1 are sensitized to heat following inflammation, but fail to drive heat hyperalgesia in the absence of TPV1 containing C-heat fibers. *Mol Pain* **6,** 58.

Latremoliere A & Woolf CJ (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain* **10,** 895–926.

Leem JW, Willis WD & Chung JM (1993). Cutaneous sensory receptors in the rat foot. *J Neurophysiol* **69,** 1684–1699.

Leith JL, Wilson AW, Donaldson LF & Lumb BM (2007). Cyclooxygenase-1-derived prostaglandins in the periaqueductal gray differentially control C- versus A-fiber-evoked spinal nociception. *J Neurosci* **27,** 11296–11305.

Liu Z-Y, Zhuang D-B, Lunderberg T & Yu L-C (2002). Involvement of 5-hydroxytryptamine(1A) receptors in the descending anti-nociceptive pathway from periaqueductal gray to the spinal dorsal horn in intact rats, rats with nerve injury and rats with inflammation. *Neuroscience* **112,** 399–407.

Lynn B & Carpenter S (1982). Primary afferent units from the hairy skin of the rat hind limb. *Brain Res* **238,** 29–43.

Magerl W, Fuchs PN, Meyer RA & Treede RD (2001). Roles of capsaicin-insensitive nociceptors in cutaneous pain and secondary hyperalgesia. *Brain* **124,** 1754–1764.

Mantyh PW (1983). Connections of midbrain periaqueductal gray in the monkey. II. Descending efferent projections. *J Neurophysiol* **49,** 582–594.

Mantyh PW & Hunt SP (2004). Setting the tone: superficial dorsal horn projection neurons regulate pain sensitivity. *Trends Neurosci* **27,** 582–584.

McMullan S & Lumb BM (2006*a*). Spinal dorsal horn neuronal responses to myelinated versus unmyelinated heat nociceptors and their modulation by activation of the periaqueductal grey in the rat. *J Physiol* **576,** 547–556.

McMullan S & Lumb BM (2006*b*). Midbrain control of spinal nociception discriminates between responses evoked by myelinated and unmyelinated heat nociceptors in the rat. *Pain* **124,** 59–68.

McMullan S & Lumb BM (2006*c*). Spinal dorsal horn neuronal responses to myelinated versus unmyelinated heat nociceptors and their modulation by activation of the periaqueductal grey in the rat. *J Physiol* **576,** 547–556.

McMullan S, Simpson DA & Lumb BM (2004). A reliable method for the preferential activation of C- or A-fibre heat nociceptors. *J Neurosci Methods* **138,** 133–139.

Meyer RA & Campbell JN (1981). Myelinated nociceptive afferents account for the hyperalgesia that follows a burn to the hand. *Science* **213,** 1527–1529.

Meyer RA, Ringkamp M, Campbell JN & Raja SN (2005). Neural mechanisms of hyperalgesia after tissue injury. *Johns Hopkins APL Tech Dig* **26,** 56–66.

Miki K, Zhou QQ, Guo W, Guan Y, Terayama R, Dubner R & Ren K (2002). Changes in gene expression and neuronal phenotype in brain stem pain modulatory circuitry after inflammation. *J Neurophysiol* **87,** 750–760.

Millan MJ (2002). Descending control of pain. *Prog Neurobiol* **66,** 355–474.

Millan MJ, Newman-Tancredi A, Audinot V, Cussac D, Lejeune F, Nicolas J, Coge F, Galizzi J, Boutin J, Rivet J, A D & Gobert A (2000). Agonist and antagonist actions of yohimbine as compared to fluparoxan at alpha(2)-adrenergic receptors (AR)s, serotonin (5-HT)(1A), 5-HT(1B), 5-HT(1D) and dopamine D(2) and D(3) receptors. Significance for the modulation of frontocortical monoaminergic transmission and depressive states. *Synapse* **35,** 79–95.

Oliva P, Berrino L, de Novellis V, Palazzo E, Marabese I, Siniscalco D, Scafuro M, Mariani L, Rossi F & Maione S (2006). Role of periaqueductal grey prostaglandin receptors in formalin-induced hyperalgesia. *Eur J Pharmacol* **530,** 40–47.

Ossipov MH, Dussor GO & Porreca F (2010). Central modulation of pain. *J Clin Invest* **120,** 3779–3787.

Ossipov MH, Suarez LJ & Spaulding TC (1989). Antinociceptive interactions between alpha , -adrenergic and opiate agonists at the spinal level in rodents. *Anesth Analg* **68,** 194–200.

Paalzow G & Paalzow L (1983). Yohimbine both increases and decreases nociceptive thresholds in rats: evaluation of the dose-response relationship. *Naunyn Schmiedebergs Arch Pharmacol* **322,** 193–197.

Palazzo E, Guida F, Gatta L, Luongo L, Boccella S, Bellini G, Marabese I, de Novellis V, Rossi F & Maione S (2011). EP1 receptor within the ventrolateral periaqueductal grey controls thermonociception and rostral ventromedial medulla cell activity in healthy and neuropathic rat. *Mol Pain* **7,** 82.

Pertovaara A (2006). Noradrenergic pain modulation. *Prog Neurobiol* **80,** 53–83.

Pertovaara A (2013). The noradrenergic pain regulation system: A potential target for pain therapy. *Eur J Pharmacol* **716,** 2–7.

Provitera V, Nolano M, Pagano A, Caporaso G, Stancanelli A & Santoro L (2007). Myelinated nerve endings in human skin. *Muscle Nerve* **35,** 767–775.

Przewłocki R & Przewłocka B (2001). Opioids in chronic pain. *Eur J Pharmacol* **429,** 79–91.

Reed RK & Rubin K (2010). Transcapillary exchange: role and importance of the interstitial fluid pressure and the extracellular matrix. *Cardiovasc Res* **87,** 211–217.

Ren K & Dubner R (1996). Enhanced descending modulation of nociception in rats with persistent hindpaw inflammation. *J Neurophysiol* **76,** 3025–3037.

Ren K & Dubner R (1999). Inflammatory models of pain and hyperalgesia. *ILAR J* **40,** 111–118.

Sandrini G, Serrao M, Rossi P, Romaniello A, Cruccu G & Willer JC (2005). The lower limb flexion reflex in humans. *Prog Neurobiol* **77,** 353–395.

Schouenborg J, Weng H & Holmberg H (1994). Modular organization of spinal nociceptive reflexes□: A new hypothesis. *Physiology* **9,** 261–265.

Sivilotti LG, Thompson SWN & Woolf CJ (1993). Rate of rise of the cumulative depolarization evoked by repetitive stimulation of small-caliber afferents is a predictor of action potential windup in rat spinal neurons in vitro. *J Neurophysiol* **69,** 1621–1631.

Soignier RD, Taylor BK, Baiamonte BA, Lee FA, Paul D & Gould HJ (2011). Measurement of CFA-induced hyperalgesia and morphine-induced analgesia in rats: dorsal vs plantar mechanical stimulation of the hindpaw. *Pain Med* **12,** 451–458.

Stanfa L & Dickenson A (1995). Spinal opioid systems in inflammation. *Inflamm Res* **44,** 231–241.

Størkson R V, Kjørsvik A, Tjølsen A & Hole K (1996). Lumbar catheterization of the spinal subarachnoid space in the rat. *J Neurosci Methods* **65,** 167–172.

Suzuki R, Morcuende S, Webber M, Hunt SP & Dickenson AH (2002). Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. *Nat Neurosci* **5,** 1319–1326.

Takano Y & Yaksh TL (1992). Characterization of the pharmacology of intrathecally administered alpha-2 agonists and antagonists in rats. *J Pharmacol Exp Ther* **261,** 764–772.

Terayama R, Dubner R & Ren K (2002). The roles of NMDA receptor activation and nucleus reticularis gigantocellularis in the time-dependent changes in descending inhibition after inflammation. *Pain* **97,** 171–181.

Tsuruoka M, Matsutani K & Inoue T (2003). Coeruleospinal inhibition of nociceptive processing in the dorsal horn during unilateral hindpaw inflammation in the rat. *Pain* **104,** 353–361.

Tsuruoka M, Tamaki J, Maeda M, Hayashi B & Inoue T (2012). Biological implications of coeruleospinal inhibition of nociceptive processing in the spinal cord. *Front Integr Neurosci* **6,** 87.

Tsuruoka M & Willis WD (1996). Descending modulation from the region of the locus coeruleus on nociceptive sensitivity in a rat model of inflammatory hyperalgesia. *Brain Res* **743,** 86–92.

Tyce GM & Yaksht TL (1981). Monoamine release from cat spinal cord by somatic stimuli: an intrinsic modulatory system. *J Physiol* **314,** 513–529.

Vanegas H & Schaible HG (2004). Descending control of persistent pain: inhibitory or facilitatory? *Brain Res Rev* **46,** 295–309.

Wang W, Wu S-X, Wang Y-Y, Liu X-Y & Li Y-Q (2003). 5-Hydroxytryptamine1A receptor is involved in the bee venom induced inflammatory pain. *Pain* **106,** 135–142.

Westlund KN, Bowker RM, Ziegler MG CJ (1983). Noradrenergic projections to the spinal cord of the rat. *Brain Res* **263,** 15–31.

Wiesenfeld-Hallin Z (1995). Neuropeptides and spinal cord reflexes. *Prog Brain Res* **104,** 271–280.

Woolf CJ & Wall PD (1986). Relative effectiveness of C primary afferent fibers of different origins in evoking a prolonged facilitation of the flexor reflex in the rat. *J Neurosci* **6,** 1433–1442.

Yeomans DC & Proudfit HK (1996*b*). Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: Electrophysiological evidence. *Pain* **68,** 141–150.

Yeomans DC & Proudfit HK (1996*a*). Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: electrophysiological evidence. *Pain* **68,** 141–150.

Yoshimura M & Furue H (2006). Mechanisms for the anti-nociceptive actions of the descending noradrenergic and serotonergic systems in the spinal cord. *J Pharmacol Sci* **101,** 107–117.

You H-J, Lei J, Sui M-Y, Huang L, Tan Y-X, Tjølsen A & Arendt-Nielsen L (2010). Endogenous descending modulation: spatiotemporal effect of dynamic imbalance between descending facilitation and inhibition of nociception. *J Physiol* **588,** 4177–4188.

Yu Z, Aihui L, Jiajia X, Lixing L, Ke R, Brian B, Ming T & Zhang R-X (2011). Involvement of spinal serotonin receptors in electroacupuncture anti-hyperalgesia in an inflammatory pain rat model. *Neurochem Res* **36,** 1785–1792.

Zhang Y, Yang Z, Gao X & Wu G (2001). The role of 5-hydroxytryptamine1A and 5-hydroxytryptamine1B receptors in modulating spinal nociceptive transmission in normal and carrageenan-injected rats. *Pain* **92,** 201–211.

Ziegler EA, Magerl W, Meyer RA & Treede RD (1999). Secondary hyperalgesia to punctate mechanical stimuli. Central sensitization to A-fibre nociceptor input. *Brain* **122 (Pt 1),** 2245–2257.

Figure legends

Figure 1: A single subcutaneous injection of CFA into the hind-paw dorsum leads to a primary mechanical but not thermal hyperalgesia.

A. Delivery of CFA (50µl) into the hind-paw dorsum produced significant oedema (one-way RM ANOVA; $p<0.0001$, $F(4,3)=33.54$, Bonferroni's post-test *$p<0.001$ compared to baseline, n=4).

B. CFA-induced inflammation was associated with a primary mechanical hyperalgesia (one-way RM ANOVA; $p<0.001$, $F(3,3)=7.13$, Bonferroni's post-test *$p<0.05$ compared to baseline, n=4).

C. CFA-induced inflammation did not however result in thermal hyperalgesia (n=4).

D. In contrast, a 50µl s.c. injection of CFA into the plantar surface of the hind-paw produced a persistent primary thermal hyperalgesia (one-way RM ANOVA; $p<0.05$, $F(3,2)=11.88$, Bonferroni's post-test, *$p<0.05$ compared to baseline, n=3).

Figure 2: A single subcutaneous injection of CFA into the hind-paw dorsum produces a transient sensitisation of withdrawal reflexes to A- and C-nociceptor activation.

A. CFA-induced inflammation of the dorsal hind-paw resulted in a transient reduction in the withdrawal threshold to A-nociceptor stimulation that resolved fully by 3 hours after CFA injection (one-way RM ANOVA, $p<0.0001$, $F(3,4)=21.22$, Bonferroni's post-test ***$p<0.001$ compared to baseline, n=5).

B. Responses to C-nociceptor activation showed that inflammation resulted in an even more transient response, resolving after 2 hours (one-way RM ANOVA, $P<0.0001$, $F(3,4)=20.39$, Bonferroni's post-test *$p<0.001$ compared to baseline, n=5). An equivalent injection of vehicle (mineral oil) had no significant effect on withdrawal thresholds to A- or C-nociceptor activation (Mineral oil group, A&B, Friedman test, $p>0.05$, n=3)

C. A larger dose of CFA (100µg/100µl) resulted in a more rapid resolution of sensitisation to A-nociceptor activation, causing a more rapid return to baseline (two-way RM ANOVA; main dose effect: $p=0.08$, $F(3,1)=3.98$, Bonferroni's post-test *$p<0.05$ between groups, n=5 for both groups).

D. A larger dose of CFA (100µg) resulted in a more rapid resolution of sensitisation to C-nociceptor activation so that withdrawal thresholds were significantly higher compared to a 50µg dose at the one hour time point (two-way RM ANOVA; Main dose effect: $p=0.0022$, $F(3,1)=19.53$, Bonferroni's post-test *** $p<0.001$ between groups, n=5 for both groups).

Figure 3: Sensitisation resulting from acute inflammation of the plantar hindpaw is unaffected by intrathecal yohimbine.

Acute CFA-induced inflammation of the plantar hind-paw resulted in a reduction in thermal withdrawal threshold that persisted for the 3 hours tested (one-way RM ANOVA; $p<0.0001$, $F(3,4)=19.30$, Bonferroni's post-test ***$p<0.001$, **$p<0.01$ compared to baseline). This was unaffected by intrathecal yohimbine at 3h following CFA (n=5 CFA, n=4 CFA+yoh).

Figure 4: Paw swelling is different following CFA injection into dorsal or plantar hindpaw.

Injection of 50µg CFA resulted in greater swelling in the plantar compared to the dorsal surface of the hindpaw. Injection of a larger CFA concentration (100µg) in the dorsal surface did not increase the swelling above that caused by 50µg. (one way ANOVA, $p<0.0001$ $F(12, 70)=59.13$, Bonferroni post-hoc tests *$p<0.05$ plantar c.f. both other groups, data shown as mean±SD).

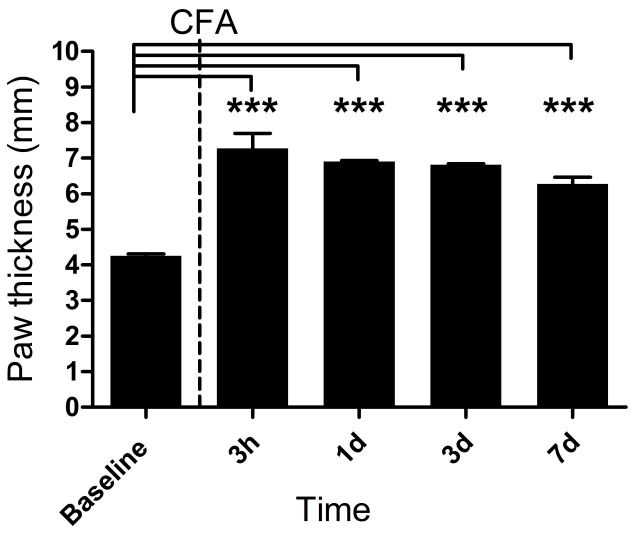
Figure 5: Blockade of spinal alpha 2 adrenoceptors reveals a rapid inhibitory control on withdrawal reflexes to A- and C-nociceptor activation during acute inflammation.

A. The loss of acute sensitisation to A-nociceptor stimulation 3 hours after CFA injection was partially reversed by the intrathecal injection of the noradrenergic alpha 2 receptor antagonist yohimbine (Mann-Whitney U, *$p<0.05$, n=7 CFA (3h), n=5 yoh).

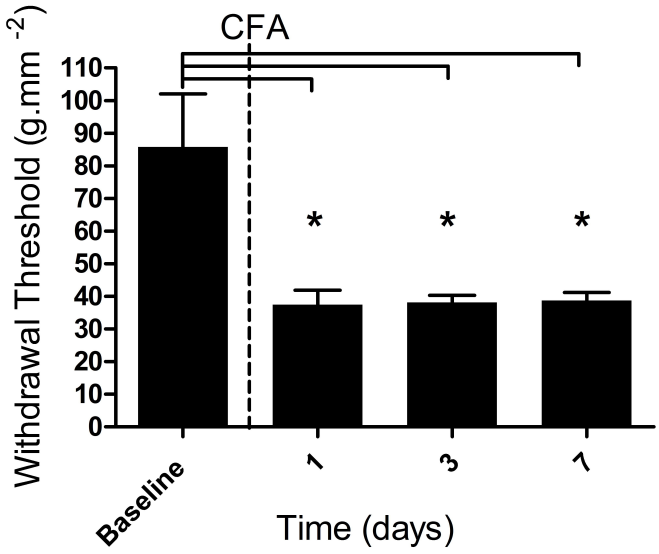
B. The loss of acute sensitisation to C-nociceptor stimulation 3 hours after CFA injection was reversed by yohimbine (Mann-Whitney U, *$p<0.05$, n=8 CFA (3h), n=5 yoh).

C. Systemic administration of the pan-opioid antagonist, naloxone, had no effect on either A-nociceptor evoked withdrawals (CFA n=5, CFA+nal n=6) or on D. C-nociceptor evoked withdrawals (CFA n=5, CFA+nal n=6).

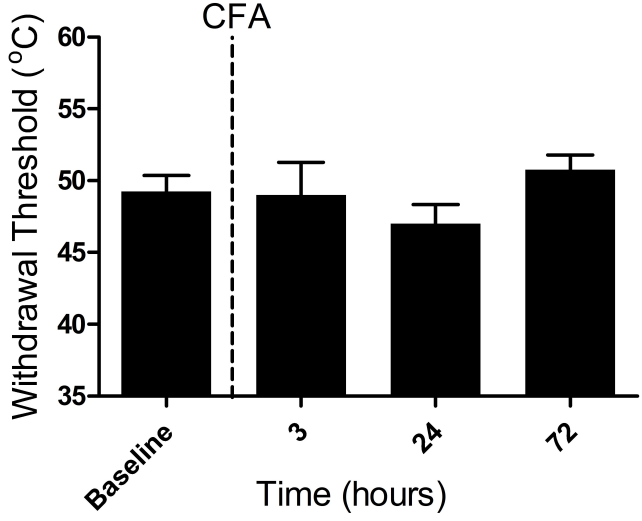
Dorsal Hind-Paw



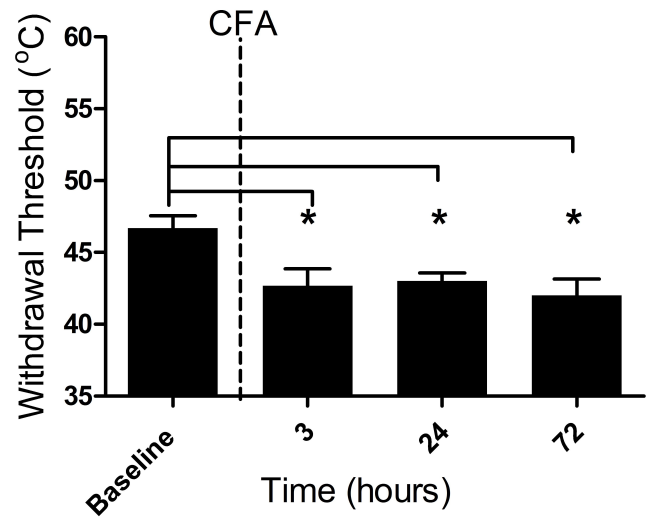
Dorsal Hind-Paw

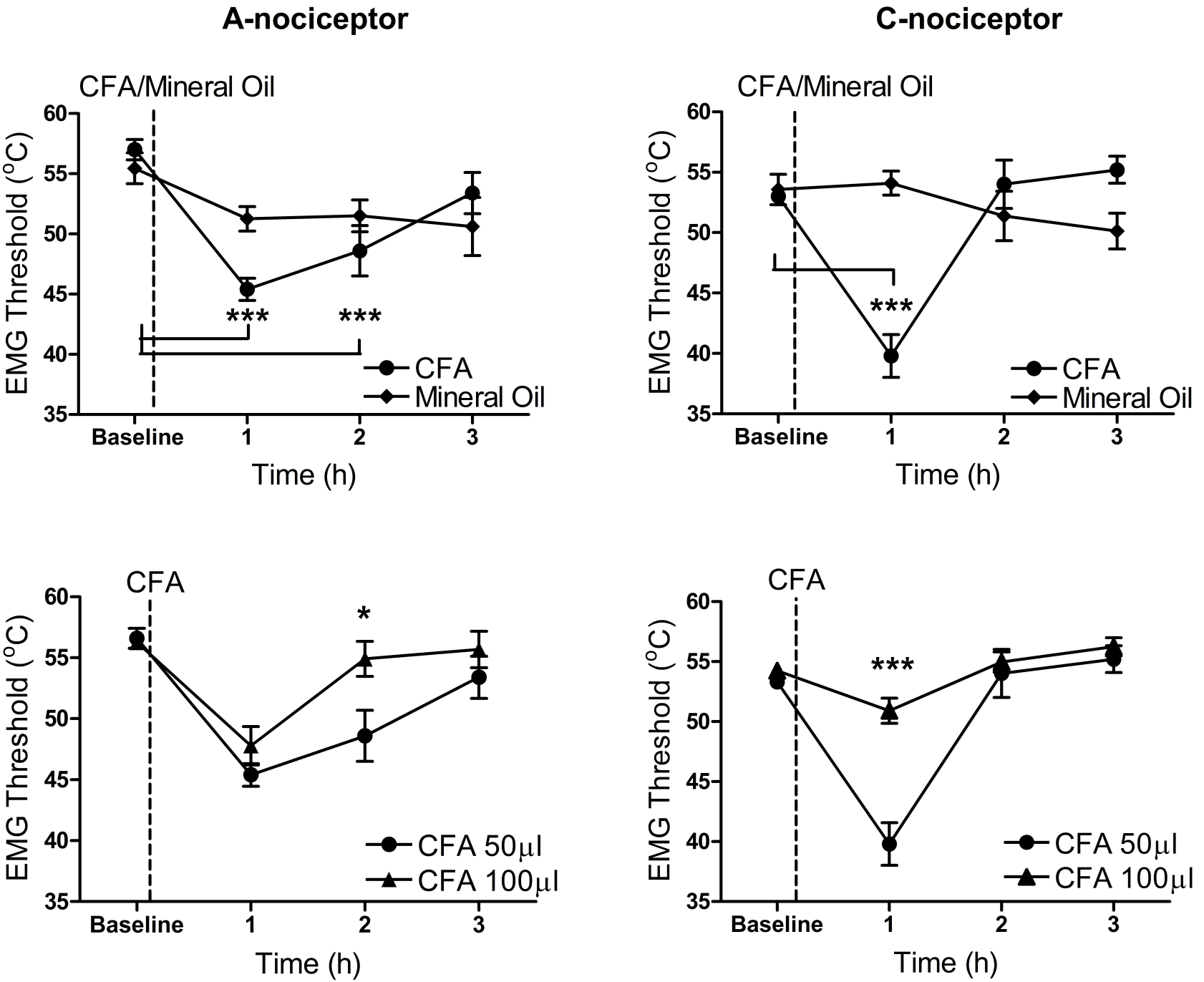


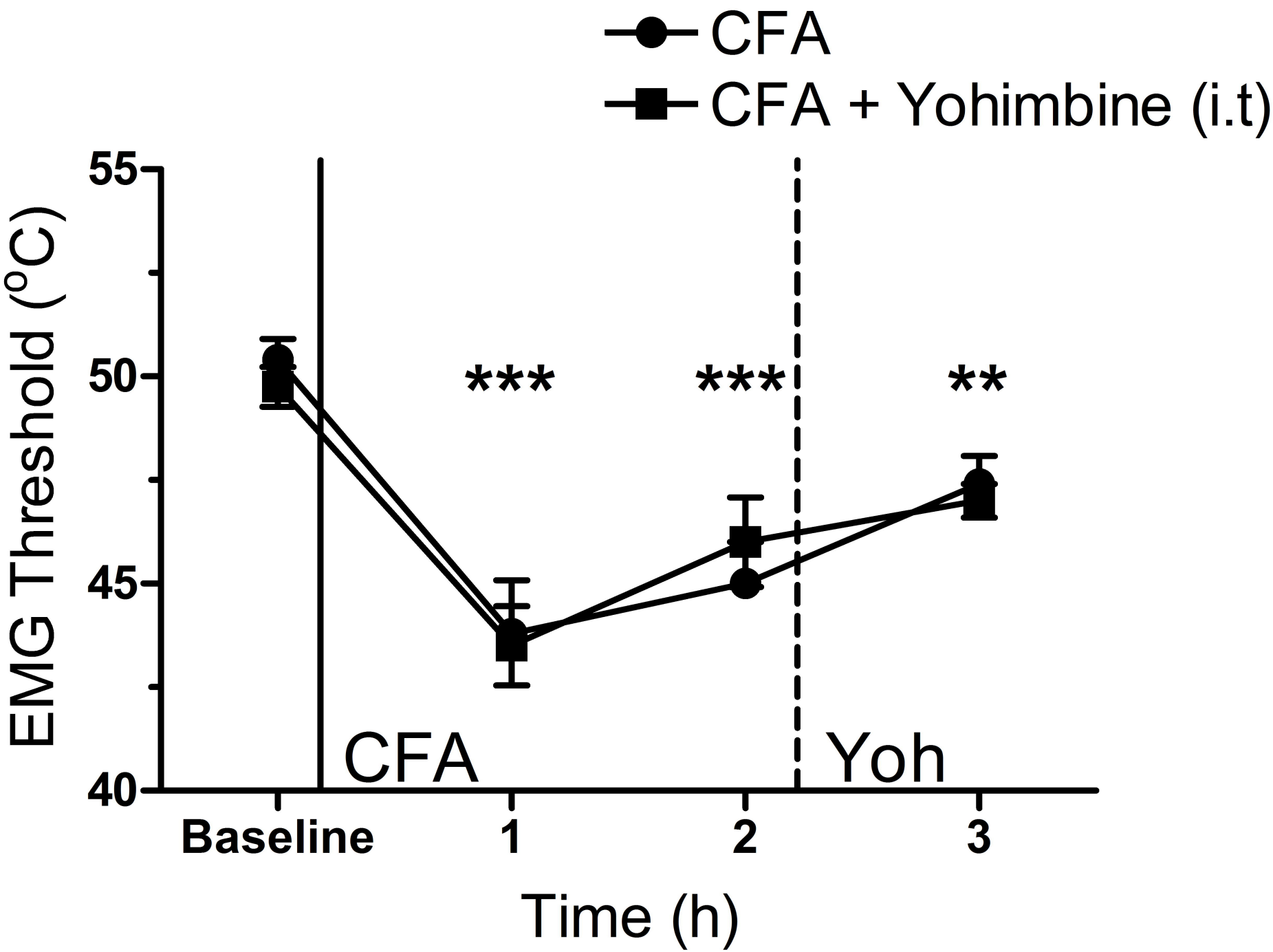
Dorsal Hind-Paw

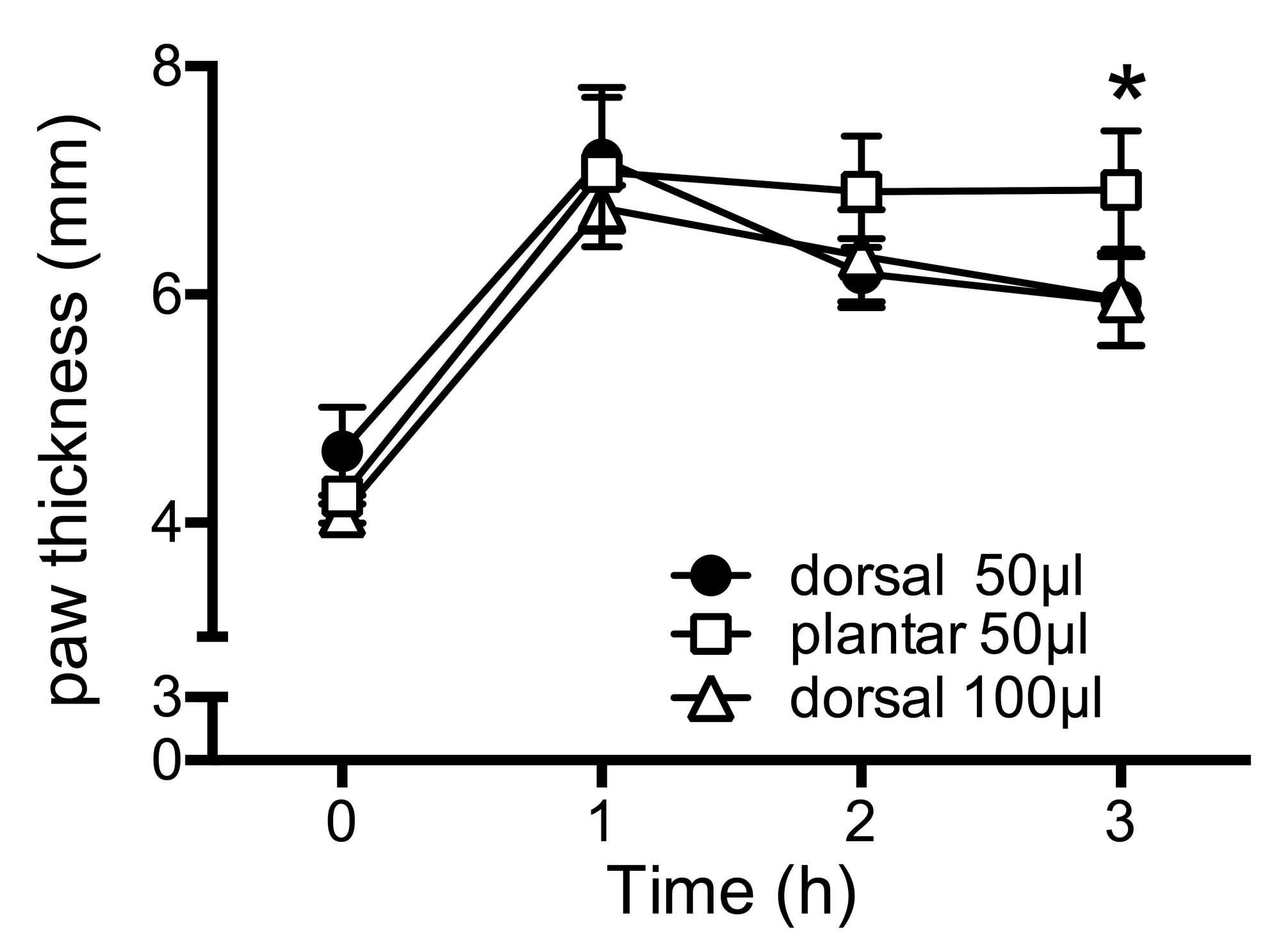


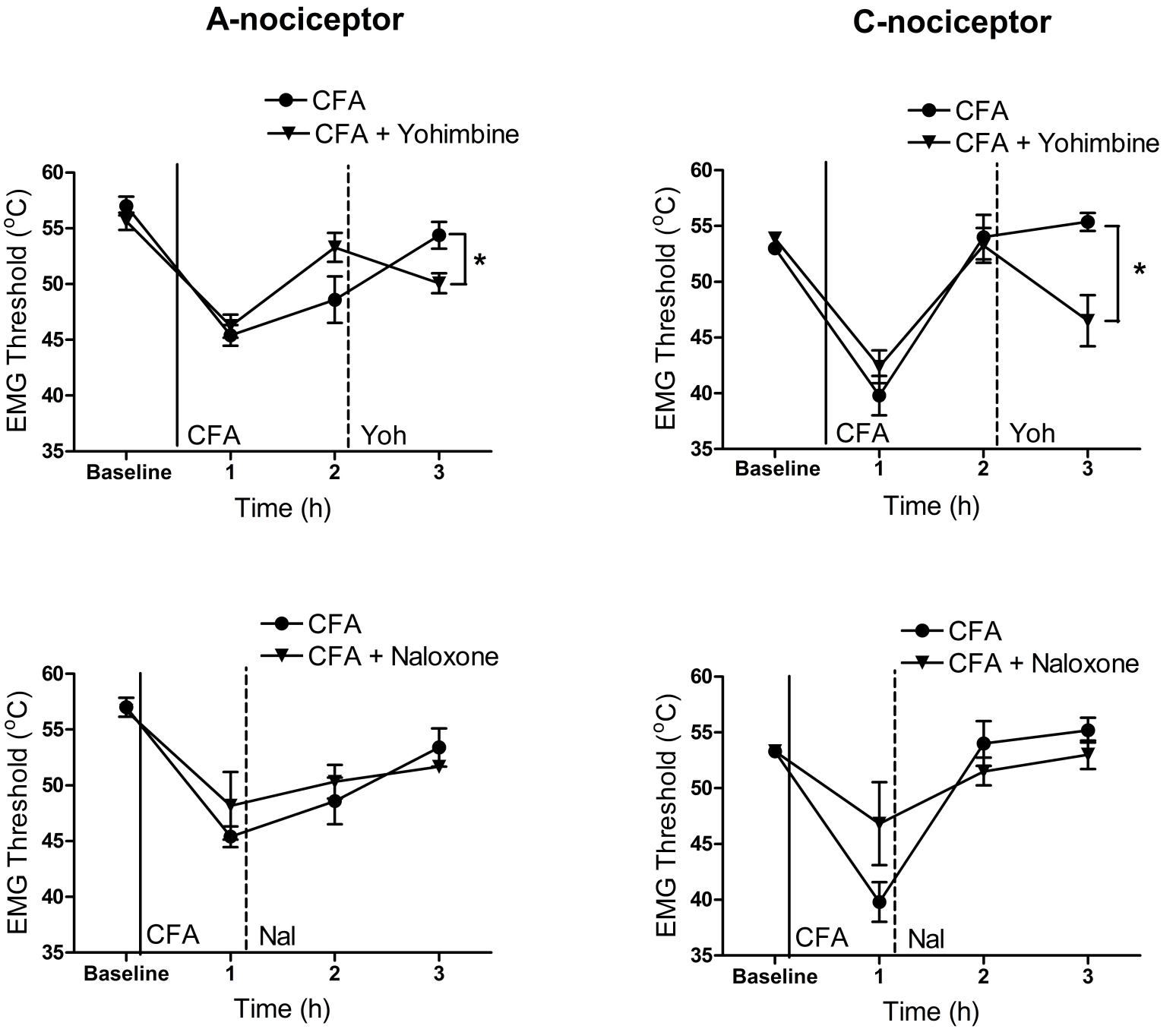
Plantar Hind-Paw




A-nociceptor
C-nociceptor
CFA/Mineral Oil
EMG Threshold (°C)
60
55
50
45
40
35
***
***
CFA
Mineral Oil
Baseline
1
2
3
Time (h)
CFA
*
***
CFA 50µl
CFA 100µl


CFA
CFA + Yohimbine (i.t)
EMG Threshold (°C)
55
50
45
40
***
***
**
CFA
Yoh
Baseline
1
2
3
Time (h)


paw thickness (mm)
8
6
4
3
0
0
1
2
3
Time (h)
*
dorsal 50µl
plantar 50µl
dorsal 100µl


A-nociceptor
C-nociceptor
CFA
CFA + Yohimbine
CFA + Naloxone
EMG Threshold (°C)
Time (h)
Baseline
1
2
3
CFA
Yoh
Nal
*
60
55
50
45
40
35