Inducible nitric oxide synthase inhibition by 1400W limits pain hypersensitivity in a neuropathic pain rat model

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Subject area: Physiology

New findings:
What is the central question of this study?
Can iNOS modulation reduce pain behaviour and pro-inflammatory cytokine signalling in a rat model of neuropathic pain?

What is the main finding and its importance?
Nitric oxide synthase based therapies could be effective for the treatment of peripheral neuropathic pain.

Abstract (250 words)

Peripheral neuropathic pain (PNP) resulting from injury or dysfunction to a peripheral nerve, is a major health problem affecting 7-8% of the population. It is inadequately controlled by current drugs, and is characterized by pain hypersensitivity which is believed to be due to sensitization of peripheral and CNS neurons by various inflammatory mediators. Here we examined, in a rat model of PNP: a) whether reducing levels of nitric oxide (NO), with 1400W, a highly selective inhibitor of inducible NO synthase (iNOS), would prevent/attenuate pain hypersensitivity, and b) the effects of 1400W on plasma levels of several cytokines that are secreted post iNOS upregulation during chronic pain states. The L5-spinal nerve axotomy (SNA) model of PNP was used, and 1400W (20mg/kg) administered intraperitoneally at 8 hour intervals for three days starting at 18 hours post-SNA. Changes in plasma concentrations of 12 cytokines in SNA rats treated with 1400W were examined using multiplex ELISA. SNA rats developed behavioural signs of mechanical and heat hypersensitivity. Compared with the vehicle/control, 1400W significantly: **a)** limited development of mechanical hypersensitivity at 66 hours post-SNA, as well as heat hypersensitivity at 42 hours and at several time-points tested thereafter, and **b)** increased the plasma concentrations of IL-1α, IL-1β, and IL-10 in the SNA rats. The findings suggest that 1400W may exert its analgesic effects by reducing iNOS and altering the balance between the pro-inflammatory (IL-1β and IL-1α) and anti-inflammatory (IL-10) cytokines and that therapies targeting NO or its enzymes may be effective for the treatment of PNP.

Introduction (500 words)

Chronic peripheral neuropathic pain (PNP) is defined as pain resulting from nerve dysfunction/lesion of the somatosensory system, and affects approximately 7-8% of the general population (Bouhassira & Attal, 2016). In humans, PNP is presented in the clinic by spontaneous/ongoing pain and hypersensitivity to normally painful stimuli (hyperalgesia) or non-painful stimuli (allodynia) (Bonica, 1990). PNP is inadequately controlled by currently available drugs, most of which lack efficacy and/or have adverse side-effects. Despite its clinical importance, the underlying mechanisms of PNP are not fully understood. However, preclinical studies using animal models of PNP, including the L5-spinal nerve axotomy (SNA) rat model (Kim & Chung, 1992), suggest that PNP is due to both sensitization of primary afferent nociceptive neurons (peripheral sensitization) and CNS neurons (central sensitization) (Campbell & Meyer, 2006; Costigan *et al.*, 2009; von Hehn *et al.*, 2012). Numerous inflammatory mediators, often termed "sensitisers", including pro-inflammatory cytokines and reactive oxygen and nitrogen species (ROS/RNS) such as nitric oxide (NO), contribute to peripheral and central sensitization (Ren & Dubner, 2010; Kim *et al.*, 2011; Schomberg *et al.*, 2012).

NO is produced by NO synthase (NOS) from L-arginine and is an important signalling molecule in cell-to-cell communication. Of the three isoforms of NOS (endothelial, neuronal and inducible), inducible NOS (iNOS) is the primary active isoform of NOS that is upregulated at early stages post injury and during chronic inflammatory processes (Gühring *et al.*, 2001; Conti *et al.*, 2007; Hamza *et al.*, 2010). The key cells that produce NO following nerve lesion are those residing in and around the injured nerves, such as activated glial cells and infiltrated macrophages that induce prolonged iNOS transcription in response to nerve injury (Aley *et al.*, 1998; Amitai, 2010; Bradman *et al.*, 2010; Benarroch, 2011). NO-has also

been shown to mediate neuronal hyperexcitability in the chronic constriction injury (CCI) pain model (Makuch *et al.*, 2013) (Mukherjee *et al.*, 2014).

Pro-inflammatory cytokines have been shown to be correlated with pain severity in chronic pain states (Marchand *et al.*, 2005; Uceyler *et al.*, 2006; Koch *et al.*, 2007b; Uceyler & Sommer, 2008; Leung & Cahill, 2010). The NO and in turn cytokines produced post-injury sensitize neurons and exacerbate hypersensitivity (von Hehn *et al.*, 2012). We hypothesized that reducing levels of NO specifically produced by iNOS at an early stage post nerve injury with 1400W, a highly selective inhibitor of iNOS (Tang *et al.*, 2007), would prevent development of pain hypersensitivity, which is believed to result from hyperexcitability of both peripheral and CNS neurons and also reduce the milieu of cytokines produced post injury which could exacerbate this hypersensitivity. This hypothesis was based on our recent findings that 1400W significantly reduced neuronal hyperexcitability in a rodent model of temporal lobe epilepsy (TLE) (Puttachary *et al.*, 2016), as well as a study by Makuch *et al.* (2013) showing 1400W to be effective in a CCI pain model.

We tested this hypothesis, in the SNA rat model of PNP that exhibits behavioural signs of allodynia and hyperalgesia, which are similar to the clinical signs observed in human patients with peripheral neuropathy (Chung *et al.*, 2004). We also examined the impact of 1400W on the plasma concentration of several cytokines which are upregulated during chronic pain states. We used 1400W, because it has been shown to be efficacious in reducing NO levels both *in vivo* and *in vitro* (Garvey *et al.*, 1997) and it is a promising agent in other neurological conditions including traumatic brain injury (Jafarian-Tehrani *et al.*, 2005), TLE (Puttachary *et al.*, 2016), and CCI whereby 1400W enhanced morphine's anti-nociceptive effects (Makuch *et al.*, 2013).

Methods

Ethical Approval

All experimental procedures were performed under a UK Home Office licence and complied with the UK Animals (Scientific Procedures) Act 1986, establishment licence X70548BEB and project licence 40/3401 and ethically approved by the University's Animal Welfare Committee. All the experiments also conform to the principles and regulations, as described in the Editorial by Grundy (2015). Adult male Wistar rats (180-280g) purchased from Charles River, UK were housed in a temperature-controlled room (22-25˚C) with food and water *ad libitum* on a 12-hour light/dark cycle in the University of Liverpool animal facilities. A total of 22 rats were used for this study; naïve + vehicle n=3, naïve + 1400W n=3, L5-SNA+ vehicle n=7and L5-SNA+1400W n=9. The investigators of this study fully understand the ethical principles under which this journal operates and that all the work carried out in this study complied with the animal ethics checklist.

Preparation of the SNA model of PNP

L5-SNA surgery was performed under deep anaesthesia in sterile and aseptic conditions. The SNA model was produced by tight ligation and transection of the left L5 spinal nerve using a modification of the procedure described originally by (Kim & Chung, 1992). Briefly, under gaseous isoflurane anaesthesia (1:1 ratio of O_2 to N_2O, 2L/min) rats were placed in a prone position, the surgical site (lumbar spine) was prepared and Videne antiseptic cream was applied. The hip bone was located and a paramedian incision was made through the lumbar epaxial muscles to expose the spinal processes of the vertebrae including the L6 transverse process. Using a sterile bone cutter, part of the L6 transverse process was removed to allow access to the L4-L5 spinal nerves. The L5 spinal nerve was exposed, a tight ligature was placed around the nerve with a 6-0 silk suture (Ethicon, Brussels, Belgium; Look, Taunton, UK) and the nerve was cut distal to the suture to prevent regeneration. Using a polysorb

thread, the muscles were sutured before the skin incision was stapled. The L5-SNA rats were randomly divided into two groups. The experimental group was treated with 1400W, and the vehicle control group received an equal volume of physiological saline. A separate sham-operated group was not used for this study because previous studies have found no significant phenotypic differences in both mechanical and thermal hypersensitivity between L5-SNA sham rats and the normal un-operated naïve rats (Ma *et al.*, 2003; Djouhri, 2006; Djouhri *et al.*, 2012). No other drugs were administered post-surgery as this was justified in the Home Office project licence on the grounds that post-operative analgesia would impact the experimental results due to the possible effects on the complex physiological processes that result in the formation of chronic pain.

Drug Administration

1400W (*N*-[[3-(Aminomethyl) phenyl] methyl]-ethanimidamide dihydrochloride (Tocris Bioscience, UK) or vehicle (physiological saline) was administered intraperitoneally at 5ml/kg beginning at 18 hours post-L5 SNA. 1400W treatment (20 mg/kg) or vehicle administration was repeated at 8 hour intervals for the first three days post-surgery. Injections were given after behavioural testing had been performed; this was to ensure that the behavioural responses were not affected by any stress or discomfort due to scruffing or injection. The optimal dose of 1400W was determined based on pilot studies of dose response experiments. The rationale for the treatment regime was based on previous studies by Parmentier *et al.* (1999) and Pearse *et al.* (2003) stating that iNOS activity develops approximately 18-24hrs post insult and the 8-hour interval between doses was chosen as it has been shown that ability of 1400W to suppress iNOS was detectable at 6hrs but inefficient at 9hrs. This study did not aim to completely block iNOS activity but rather to suppress the pathological levels of iNOS mediated NO production following nerve injury. The dosage and

treatment course have been shown to be effective in iNOS suppression and in reducing neuropathological outcomes in various experimental models (Garvey *et al.*, 1997; Parmentier *et al.*, 1999; Puttachary *et al.*, 2016).

Behavioural sensory testing

All of the rats used in the present study maintained good health and normal levels of exploratory, feeding, and grooming activities, and their weight gain during the course of the experiment was indistinguishable from naïve rodents. The behavioural tests were performed in specialised behaviour testing chambers (Ugo Basile, Italy). All animals were acclimatised to the cages and nociceptive responses performed once exploratory and grooming behaviours had stopped. We assessed responses to both mechanical and heat stimuli to assess mechanical and thermal hypersensitivity, the two forms of evoked pain behaviours that are routinely observed in clinical pain settings (Kim *et al.*, 1997; Backonja & Stacey, 2004). The stimuli were applied to the mid-plantar surface of the ipsilateral hind paw (L4 dermatome), avoiding the footpads. Habituation was performed 2–3 days before testing for the baseline pain values (pre-SNA). Assessments for the baseline values were carried out 1 day before SNA surgery. Pain behavioural tests were also conducted every 8 hours starting at 18 hours post-L5 SNA for 3 days, followed by every 24 hours for 10 days. The researcher who carried out the behavioural studies was blinded to the drug treatment and comparisons between pain-related behaviours were made in 2 groups only: 1400W-treated and vehicle-treated L5 SNA rats. For each behavioural test, each stimulus was applied four times and the Mean±SD values for each time point was calculated for all the groups. The two forms of evoked pain (mechanical and heat hypersensitivity) were assessed by measuring the following parameters:

a) Withdrawal threshold to mechanical stimulus

The automated von Frey/dynamic plantar electronic aesthesiometer (UgoBasile, Italy) was used to evaluate mechanical hypersensitivity (mechanical allodynia). A blunt probe was applied at increasing force intensities, from 0-50g over 15 seconds, through an elevated mesh floor, to the plantar surface of the animal's hind paw whilst the rat was held in a Perspex cage. Tests were repeated four times and the mean of these trials was used for each time point for each rat. The withdrawal threshold was recorded in grams (g). Mechanical hypersensitivity was inferred from the decreased mean paws withdrawal thresholds to a mechanical stimulus as described previously (Weng *et al.*, 2012).

b) Withdrawal latency to noxious heat

As described previously (Djouhri, 2006; Weng *et al.*, 2012), heat hypersensitivity/hyperalgesia was inferred from a decrease in the mean paw withdrawal latency (in seconds) to the heat stimulus using a Hargreaves analgesiometer (Ugo Basile, Comerio, Italy). Each rat was placed on a 2mm thick glass floor under which the laser heat source was aligned. The thermal stimulus (50^{o}C) was applied from a concentrated circular 9mm round heat source. The withdrawal response time (latency) to the heat stimulus was measured in seconds. The onset of heat stimulus activated a timer that stopped automatically when the evoked paw withdrawal was detected. Three measurements of latency were taken and averaged for each hind paw and for each rat. To minimize sensitization, the rodent hind paws were tested alternately with 5 minute intervals between stimuli on the same hind paw.

Plasma cytokine expression profiles

At the end of the experiments, all animals were euthanized with an overdose of pentobarbitone (60 mg / kg, i.p.). Under terminal anaesthesia, the caudal vena cava was transected to collect blood for the cytokine ELISA. Immediately afterwards, transcardiac perfusion was performed with 4% paraformaldehyde in PBS in 0.1M (PBS-PFA) for tissue

collection for studies not reported here. Ideally, repeated samples would have been taken throughout the study but this was not allowed due to the nature of the Home Office licence and the volume of blood needed for the assays. The blood was immediately placed in plasma separation tubes containing 3 I.U Heparin/ml of blood. All samples were stored on ice, protected from light, and then centrifuged at 2000*g* for 10 minutes. The supernatant containing the plasma was separated and immediately frozen at −20°C prior to cytokine profiling. A rat pro-inflammatory multiplex ELISA kit (Qiagen, UK) was used to determine plasma concentrations of 12 inflammatory cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNF-α, GM-CSF and RANTES) which are known to be involved in pro- or anti-inflammatory processes following injury to the nervous tissue (Schäfers *et al.*, 2003; Sung *et al.*, 2004; Zhang & An, 2007; Wang *et al.*, 2012). The multiplex ELISA was carried out as per the manufacturer's instructions with appropriate positive and negative controls. The multiplex ELISA plates were read in a SPECTROstar Nano Microplate Reader (BMG LabTech, Germany) within 30 minutes of adding the stop solution. The optical density (OD) values were obtained and once blank corrected, Mean±SD was calculated for each sample and then appropriate statistical analysis performed.

Statistical Analysis

The behavioural data were normally distributed and were therefore, expressed as mean ± SD. A repeated measures ANOVA followed by a Tukey Pairwise Comparison was used to analyse the behavioural data for each of our 5 time points with a full 16 animal datasets (Minitab Inc, PA, USA). For plasma cytokine analysis, a general linear model followed by a Tukey`s Post-hoc test was performed (Minitab Inc, PA, USA). Statistical significance was

defined as $P \leq 0.05$ and the significance level is indicated on the graphs as follows: *P< 0.05; **P < 0.01, and ***P < 0.001.

Results

1400W limits development of mechanical hypersensitivity after L5-SNA

To determine whether reducing levels of iNOS-mediated NO levels with 1400W would attenuate mechanical hypersensitivity/allodynia associated with nerve injury, we measured the paw withdrawal thresholds (PWT) to a mechanical stimulus (using an automated von Frey type system, see Methods). The data were collected before and at several time points after L5-SNA in SNA rats treated with 1400W (1400W group), and in vehicle-treated SNA rats. There was a significant effect of treatment ($F_{(1,56)}$ = 8.86; P<0.01), and time ($F_{(3,56)}$ = 13.67; P<0.001)) on mechanical hypersensitivity and a significant interaction between treatment and time ($F_{(3,56)}$ = 6.21; P<0.01). Comparison of PWT values at various time points (18, 42, 66, 90, 162 and 258 hours) after the L5-SNA in the vehicle group with the pre-operated (pre-SNA) values, showed significant decreases (P<0.05) in the mean PWT at all the time points (Fig. 1) indicating development of mechanical allodynia in vehicle-treated SNA rats. Interestingly, the mean PWTs in the 1400W group were significantly higher at 66 hours post treatment compared to those in the vehicle group. The mean PWT at the latest point tested (258 hours) was also similar to the pre-axotomy (pre-SNA) value. However, as shown in Fig. 1, there was no significant change in the mean PWT at other time points (18, 42, 90, 162 hours) in the drug treatment group when compared with the vehicle group.

1400W limits development of heat hyperalgesia after L5-SNA

Comparisons of the mean paw withdrawal latency (PWL) values between the vehicle treated at the different time points post-surgery and the pre-operated (pre-SNA) values show that at

all the time points tested (Fig. 2) there were significant decreases ($P<0.05$) in the mean PWL, indicating development of heat hypersensitivity/hyperalgesia in the vehicle-treated SNA rats. 1400W significantly increased the mean PWL at several time points (42, 66, 162 hours) when compared with the vehicle group (Fig. 2). Overall, there was a significant effect of treatment $F_{(1,56)} = 110.33$; $P<0.001$), and time ($F_{(3,56)} = 2.73$; $P\leq0.05$) on mechanical hypersensitivity and a significant interaction between treatment and time ($F_{(3,56)} = 3.64$; $P<0.05$).

1400W treatment increases the plasma concentrations of IL-1α, IL-1β, and IL-10 cytokines in L5-SNA rats

Having established that 1400W limits heat and mechanical hypersensitivity in SNA rats, we examined our hypothesis that 1400W may exert its analgesic effects by altering the levels and ratios of pro- and anti-inflammatory/nociceptive cytokines. To investigate this, we analysed plasma concentrations of 12 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNF-α, GM-CSF and RANTES).

Eight of the 12 measured cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, 1L-12 and IFN-γ) were significantly upregulated post axotomy when compared to naïve rats as shown in figure 3 ($P<0.05$). In addition to this, when examining the L5-SNA treated rats, 1400W treatment post-surgery caused a significant increase in the plasma concentrations of IL-1α, ($P<0.05$), IL-1β ($P<0.01$) and IL-10 ($P<0.01$) when compared with the vehicle group.

Discussion

In this study, we investigated for the first time, whether the selective iNOS inhibitor, 1400W limits pain hypersensitivity in the rat L5-SNA model of PNP. We found that 1400W administered intraperitoneally at 8 hour intervals starting at 18 hours post-SNA is more effective in preventing development of heat hypersensitivity/hyperalgesia than mechanical

hypersensitivity/allodynia. Whilst the L5-SNA model itself is known to induce the release of several inflammatory cytokines, as in human counterparts (Strong *et al.*, 2012), but the interesting aspect of this study is that compared to the vehicle-treated SNA rats, 1400W increased the plasma levels of the anti-inflammatory cytokine (IL-10) and the pro-inflammatory cytokines (IL-1α and IL-1β) in the SNA rats.

1400W limits the development of both mechanical and heat hypersensitivity in SNA rats.

The L5-SNA model used in the present study is one of the most widely used models of PNP; numerous studies have shown this model to exhibit long-lasting (more than 8 weeks) behavioural signs of mechanical allodynia and heat hyperalgesia (Chung *et al.*, 2004; Jaggi *et al.*, 2011). As reported previously (Djouhri *et al.*, 2012), these two evoked pain behaviours were indicated by significant decreases in the mean PWT from a mechanical stimulus, and in the mean paw withdrawal latency from a noxious heat stimulus respectively. The most significant finding of the present study is that 1400W prevented the development of both heat hypersensitivity/hyperalgesia and mechanical hypersensitivity/allodynia albeit to different extents. Fluctuations in the release of pro- and anti-inflammatory cytokines and the altered balance between these cytokines at different time points could underlie why we observed a significant reduction at only one time point in the mechanical hypersensitivity study. A limitation of this study was that cytokine concentrations were only measured at the end point as outlined in the methods and it would be very interesting to explore the temporal profile of all the 12 cytokines measured over time. This pain hypersensitivity is believed to result, at least partly, from hyperexcitability of primary afferent dorsal root ganglion (DRG) neurons (von Hehn *et al.*, 2012). However, since in the L5-SNA model, the axotomized L5-DRG neurons are disconnected from their peripheral target in the hind paw, the anti-hyperalgesic and anti-allodynic effect of 1400W, in this model, must involve excitability modulation

(hyperexcitability) of the adjacent L4-DRG neurons (whose nerve fibres intermingle with the degenerating fibres of the L5-DRG neurons in the partially injured peripheral nerve) which are necessary for transmission of evoked pain signals to the CNS.

We have previously demonstrated that axotomy increases the levels of nNOS in neurons, and iNOS in satellite glia cells (Schwann cells) and the infiltrated macrophages ($CD68^{+ve}$ cells) in the DRGs (Bradman *et al.,* 2010). iNOS levels have also been shown to be significantly upregulated during 12-24 hours post-nerve injury and to persist for a long period in both DRGs and the spinal cord (Iadecola *et al.,* 1995; Levy & Zochodne, 1998; Levy *et al.*, 1999; Parmentier *et al.,* 1999; Perez-Asensio *et al.,* 2005; De Alba *et al.,* 2006; Martucci *et al.*, 2008). Although nNOS has a protective role in axotomized DRG neurons (Thippeswamy & Morris, 1997; Thippeswamy & Morris, 2002; Thippeswamy *et al.*, 2004), the excessive NO produced from glia and infiltrated macrophages after peripheral nerve injury is known to sensitize both injured L5- and neighbouring *uninjured* L4-DRG neurons, as well as spinal cord neurons (Scholz & Woolf, 2007). Given that we have previously shown 1400W to suppress hyperexcitability of brain neurons for a prolonged period when administered soon after brain insult (Puttachary *et al.,* 2016), it is possible that 1400W exerts its analgesic effects by reducing the sensitizing effects of NO on the *uninjured* L4-DRG neurons. However, a central mechanism of 1400W action by way of interfering with the central sensitization processes cannot be excluded, since we have demonstrated that 1400W crosses the BBB (Puttachary *et al.*, 2016).

Our findings that 1400W reduced neuropathic pain behaviours in the SNA rats are consistent with those of previous studies showing that blocking iNOS by intrathecal administration of 1400W prior to induction of inflammation reduced inflammatory hyperalgesia in two experimental rat models of inflammatory pain (Tang *et al.*, 2007), and that 1400W reduced inflammatory pain in a rat model of arthritis when administered at a prophylactic dose (Rocha

et al., 2002). The dosing regimen of 1400W tested in the present study is based on 1400W's pharmacokinetic profile, and on the previous studies showing 1400W to be inefficient if given at intervals of longer than 9 hours (Parmentier *et al.*, 1999; Perez-Asensio *et al.*, 2005).

1400W increases plasma concentrations of inflammatory cytokines in SNA rats.

In the present study, we have investigated the plasma concentrations of 12 commonly known cytokines in SNA rats treated with 1400W because nerve injury-induced cytokines are expected to be released into the circulation. 1400W has previously been shown to ameliorate the pathogenesis of numerous neurological conditions (Parmentier *et al.*, 1999; Pearse *et al.*, 2003; Jafarian-Tehrani *et al.*, 2005), and with its similar structure and molecular weight to L-arginine suggest it is BBB permeable. Indeed, we have previously demonstrated that 1400W can cross the BBB and exert its effects in rats, by reducing the serum albumin and 3-nitrotyrosine (3-NT) levels in the hippocampus in the rat model of TLE (Puttachary *et al.*, 2016). Another study by Ryu and McLarnon (2006) outlined how 1400W can prevent BBB leakiness and reduce 3-NT levels in the hippocampus and is effective in modulating the target molecules within the brain and therefore able to cross BBB. The effects of 1400W reported in this study also imply that this selective inhibitor of iNOS may also act, at least in part, in the periphery, although the plasma studies carried out here investigated the systemic effects that 1400W may have. We found that out of the 12 cytokines examined, the levels of IL-1α, IL-10 and IL-1β were significantly elevated in the 1400W treated SNA rats. One possible mechanism for the effectiveness of 1400W in limiting the development of heat hyperalgesia and mechanical allodynia in SNA rats is by altering the balance between the pro-and and anti-inflammatory cytokines. This is because both IL-1α and IL-10 are typically thought to have anti-nociceptive effects (Vale *et al.*, 2003; Wang *et al.*, 2012; Zychowska *et al.*, 2013;

Willemen *et al.*, 2014). However, IL-1β is a widely reported pro-inflammatory mediator/pro-nociceptive (Zychowska *et al.*, 2013).

The roles of IL-1α and IL-1β in nociceptive signalling have not been well characterised, but IL-1α expression levels are upregulated following nerve injury. However, a study by Mika *et al.* (2008) found that contrary to what might be expected with a pro-inflammatory cytokine, IL-1α administration had both anti-allodynic and anti-hyperalgesic properties (Rothman & Winkelstein, 2010; Kras *et al.*, 2014). IL-1α and IL-1β have both been shown to cause increased expression of substance P in DRG neurons *in vitro* (Skoff *et al.* (2009). These findings are difficult to reconcile with the proposed anti-nociceptive and pro-inflammatory role for IL-1α (see above) because substance P is involved in the generation of neurogenic inflammation and is widely considered to be an algogenic substance, although low doses of substance P have been reported to produce analgesia in a rodent pain model (Frederickson *et al.* (1978). The results presented in this study of increased plasma concentrations of IL-1β in SNA rats, compared with naïve rats, are consistent with those of previous studies of peripheral nerve injury (Sweitzer *et al.*, 1999; Sommer & Kress, 2004; Zhang & An, 2007; Uceyler & Sommer, 2008). Our findings in this study show elevated levels of IL-1β in SNA rats treated with 1400W which is unexpected because IL-1β is considered as a pro-nociceptive factor (Zychowska *et al.*, 2013), and its upregulation is expected to exacerbate and not ameliorate neuropathic pain symptoms. However, IL-1β has been shown by some to also possess anti-nociceptive properties (Souter *et al.*, 2000).

Our findings of increased plasma concentrations of IL-10 in SNA rats are consistent with those of previous studies showing increased plasma concentrations of IL-10 in human patients with neuropathic pain and in rodent models of neuropathic pain (Koch *et al.*, 2007a; Khan *et al.*, 2015). IL-10 is believed to be effective in preventing or reducing pain in animal models by suppressing the synthesis of the pro-inflammatory mediators such as IL-1β and

TNF-α (Milligan *et al.* (2005); Soderquist *et al.* (2010); Clark *et al.* (2013); Zychowska *et al.* (2013), and to mediate both anti-inflammatory and anti-nociceptive functions (Austin & Moalem-Taylor, 2010). Based on these findings, and on our findings of elevated levels of IL-10 in SNA rats treated with 1400W, one mechanism by which 1400W could prevent development of heat hyperalgesia and mechanical allodynia after L5-SNA is by way of increasing IL-10 levels in the microenvironment of *uninjured* L4 DRG neurons. These could in turn reduce levels of pro-inflammatory mediators and therefore the neuroinflammation associated with SNA. It is noteworthy, however, that any given cytokine may behave as a pro- as well as an anti-inflammatory cytokine depending on various factors including its local concentration, its target cell, or even the experimental model (Cavaillon, 2001). The ultimate outcome of any cytokine-signalling is also dependent on the type of the cytokine receptor being expressed and activated. For example, activation of IL-1 type I receptor mediates the inflammatory action of IL-1, whereas type II receptor activation supresses IL-1 binding activity (Arend, 1991; Kuno & Matsushima, 1994) suggesting that mere upregulation of ligands does not necessarily result in change of target cell functions. But in light of these data, and the behavioural study carried out on these L5-SNA treated rats, it is plausible that 1400W may function by increasing the anti-nociceptive mediators (IL-1α and IL-10) to counteract and balance the neuro-immune response and in turn reduce neuronal hypersensitivity.

In conclusion, NO is one of the most potent known pro-inflammatory species. We found that inhibiting NO synthesis with a highly specific iNOS inhibitor, 1400W, limits the development of heat and mechanical hypersensitivity in the L5-SNA model of PNP. The measurement of concurrent increases in IL-1α, IL-1β, and IL-10 concentrations in the plasma of 1400W treated rats suggests that iNOS contributes to the pathophysiology of PNP and that 1400W may exert its analgesic action by altering the balance between the pro- and anti-inflammatory cytokines. These data suggest that therapies that target NO or its enzymes may be effective for treatment of PNP.

Competing interests

There are no competing interests in relation to this study.

Author contributions

All experiments were conducted at the University of Liverpool, UK

CAS, RBJ, LD and TT all contributed to the design, analysis and the drafting of the work outlined in this manuscript. Similarly all authors approved the final version and agree to be accountable for all aspects of the work. TT and LD received funding for this study from the Pain Relief Foundation, UK.

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Figure legends

Figure 1. Effects of 1400W on SNA-induced mechanical hypersensitivity/allodynia. Intraperitoneal administration of 1400W at 8 hour intervals starting at 18 hours post-surgery limited the development of mechanical hypersensitivity as evidenced by a significant reduction in the mean paw withdrawal threshold (PWT) to a mechanical stimulus at 66 and 258 hours post-SNA compared with vehicle (*n*=9 for 1400W group; n=7 for the vehicle group; $P<0.001$ repeated measures ANOVA with Tukey post hoc test).

Figure 2. Effects of 1400W on SNA-induced heat hypersensitivity/hyperalgesia. Intraperitoneal administration of 1400W at 8 hour intervals starting at 18 hours post-surgery prevented development of heat hypersensitivity as evidenced by a highly significant reduction ($P<0.001$) in the mean paw withdrawal latency (PWL) to a heat stimulus at 3 of the time pointed tested post-SNA..

Figure 3. Plasma concentrations of cytokines in naïve and L5-SNA rats. The data in this figure are presented as median, with the median values of plasma concentrations of cytokines being represented with the horizontal lines within the rectangular boxes. The boxes represent the interquartile range of the values of measured cytokines, whereas the whiskers span minimum to maximum measured values including outliers. For all cytokines measured, there were no significant changes in cytokine concentrations between the naïve + vehicle and the naïve + 1400W however in the pain states (denoted by L5-SNA underneath), 1400W caused a significant increase in the median levels of the pro-inflammatory cytokines IL-1α ($P<0.05$) and IL-1β ($P<0.01$) as well as a highly significant increase ($P<0.001$) in the anti-inflammatory cytokine IL-10 compared with the vehicle group. The figure also shows how 8 of the 12 cytokines examined were significantly higher in L5-SNA rats compared to the naïve rodents. The statistical analysis was carried out using the General Linear Model and a Tukey post hoc test (significance denotes as $p<0.05$, naïve+vehicle n=3, naïve+1400W n=3, L5-SNA+vehicle n=6, L5-SNA+vehicle n=6).

Figure 1

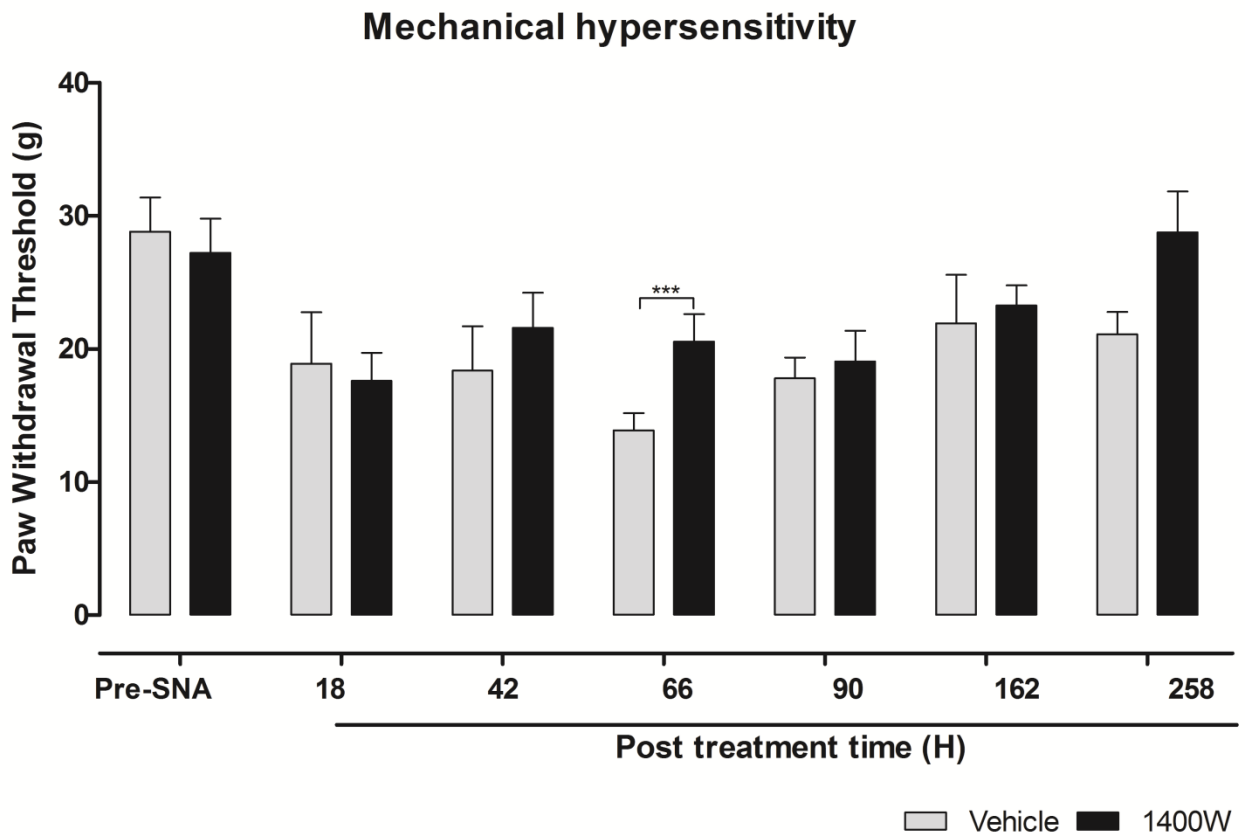

Mechanical hypersensitivity
Paw Withdrawal Threshold (g)
40
30
20
10
0
***
Pre-SNA
18
42
66
90
162
258
Post treatment time (H)
Vehicle
1400W

Figure 2

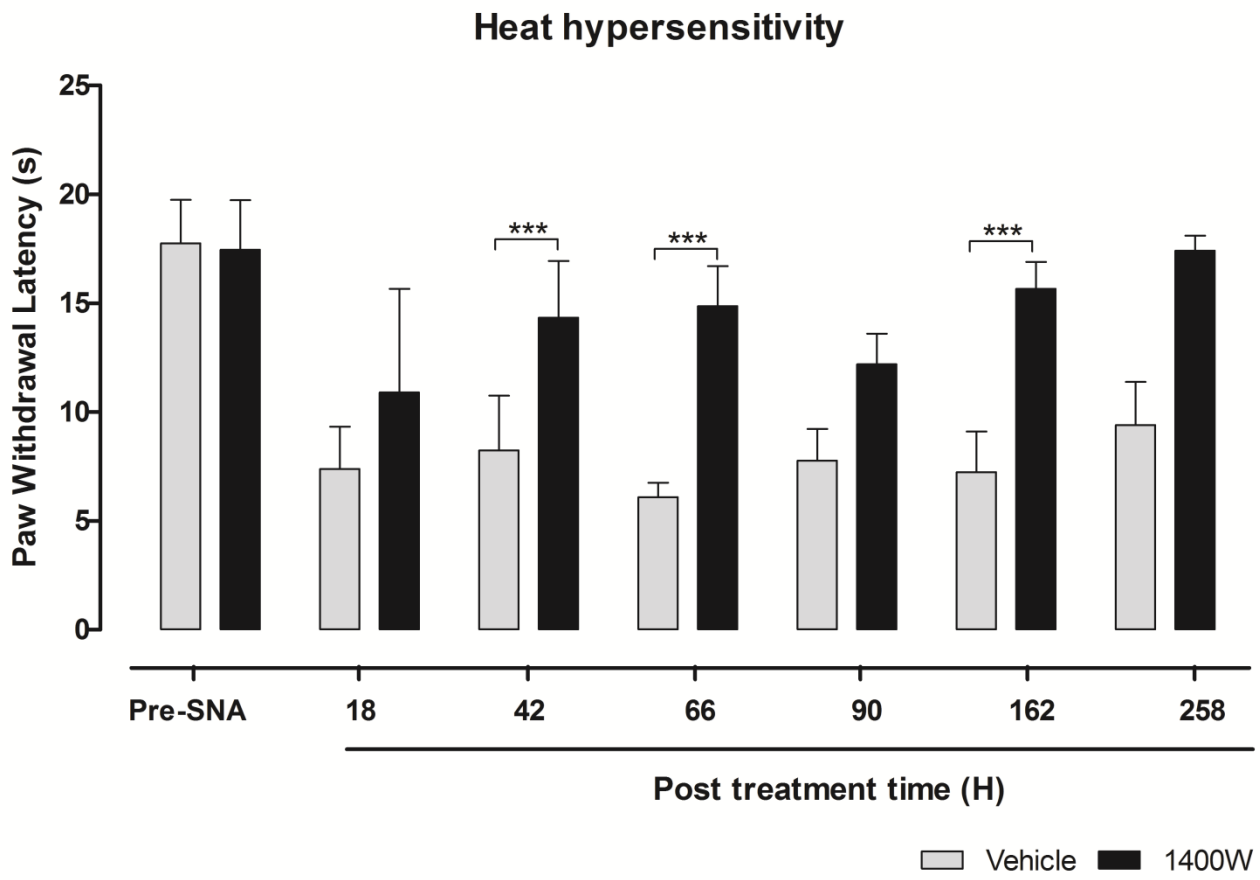
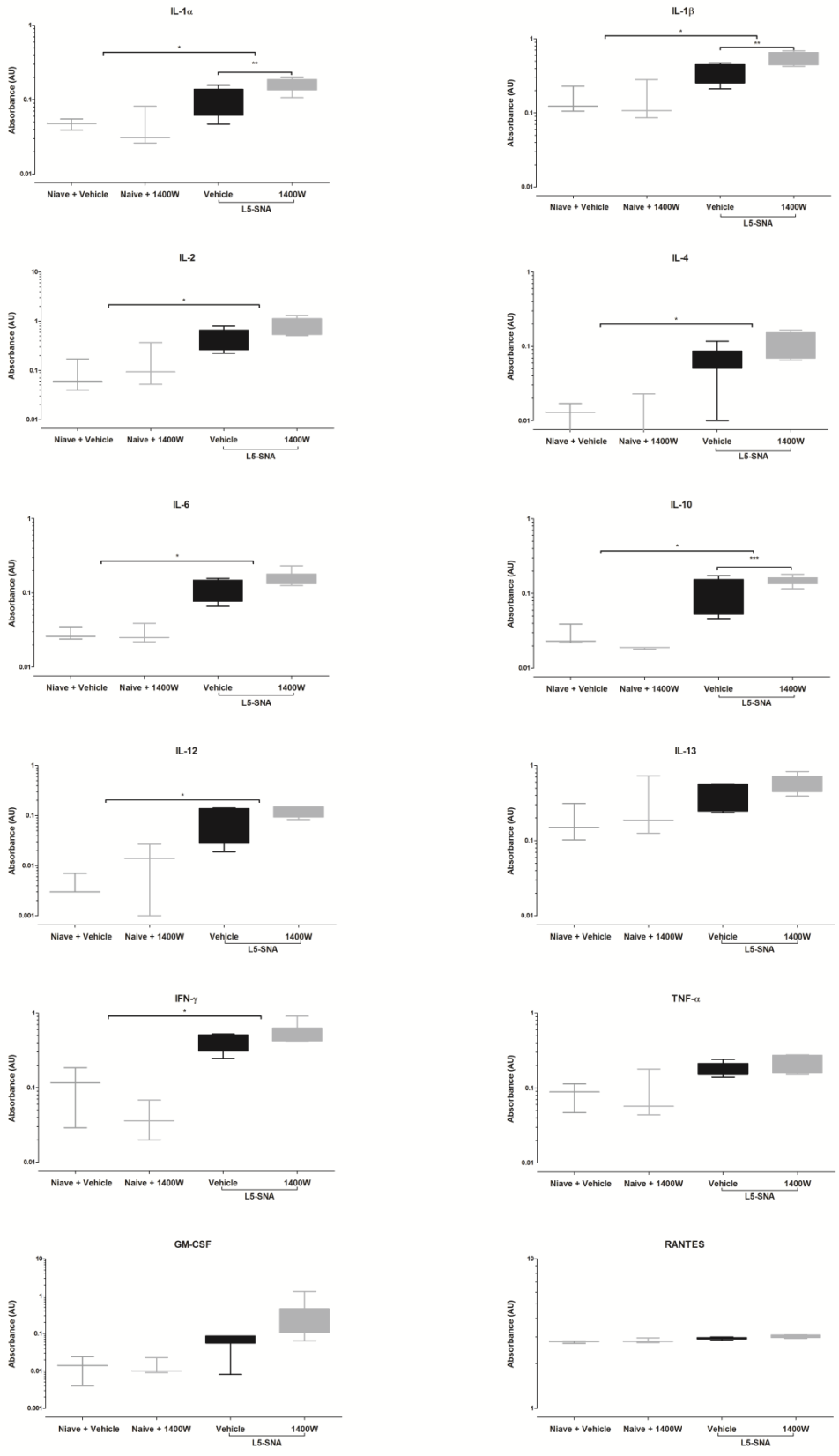

Heat hypersensitivity
Paw Withdrawal Latency (s)
25
20
15
10
5
0
***
***
***
Pre-SNA
18
42
66
90
162
258
Post treatment time (H)
Vehicle
1400W

Figure 3


IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-10
IL-12
IL-13
IFN-γ
TNF-α
GM-CSF
RANTES
Absorbance (AU)
Niave + Vehicle
Naive + 1400W
Vehicle
1400W
L5-SNA