Investigations in acute and chronic allodynia:

Using human psychophysics, sensory analysis, glial modulation & functional proteomics in the dorsal horn of the spinal cord

Thesis for Doctor of Philosophy (Ph.D.) degree

By

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Name: **Sumaiya Shaikh**

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List of abbreviation

CT: unmyelinated C- tactile fibres (in humans)
C-LTMR: unmyelinated C- low threshold mechanoreceptors (in animals)
A-LTMR: myelinated or large diameter low threshold mechanoreceptors
LTMR: Low threshold mechanoreceptors
HTMR: High threshold mechanoreceptors
VGLUT3: Vesicular glutamate transporter 3 (C-LTMR marker)
ATP: Adenosine triphosphate
WT: Wild type mice (non-genetically modified)
TRPV1: Transient receptor potential cation channel subfamily V member 1
CaV3.2 & CaV3.3: Calcium channels, voltage-dependent, T type, alpha 1 subunit, or CACNA1 (H/I)
TAFA4: Chemokine-like protein (or FAM19A4) that tags C-LTMRs
GINIP: Gαi-interacting protein, modulates GABAergic inhibitory function
TH: tyrosine hydroxylase
GFRα2+ : Glial cell-derived neurotrophic factor family receptor
IB4: Isolectin B4
PKCγ: gamma isotype of protein kinase C
C-Fos: a proto-onco gene (a marker of neuronal activity),
Pax2: Paired box gene 2 (a marker for GABAergic neurons)
CTb: B subunit of cholera toxin
ERK: Extracellular Signal-regulated Kinase-1
MAPK: Mitogen-activated protein kinases
KV4.2: A-type voltage gated potassium channel

Overview and Structure

In many chronic pain pathologies, stimuli that are normally non-painful to healthy humans, such as light touch or small decrements in temperature, can cause painful responses – a phenomenon called ***allodynia***. The majority of research on tactile allodynia has focused on myelinated low threshold A mechanoreceptors (A-LTMR) that are otherwise known to mediate innocuous mechano-sensation (1, 2). Recently, there is a growing recognition of the contribution of more than one class of low threshold fibres to pain processing, i.e. unmyelinated low threshold C mechanoreceptors (C-LTMRs), which are believed to be responsible for affective touch processing. (3-6).

The first evidence of the contribution of C tactile fibres (CTs), the human counterpart of C-LTMRs, to cutaneous allodynia was shown in human models of rapid-onset skin and muscle pain as well as delayed-onset muscle soreness (3-9). In addition, recent studies in transgenic mice, in both acute and chronic pain models, have provided molecular mechanisms at the spinal level, which functionally correspond to the tactile and cold allodynia perceived in human subjects (10-13). In the last few years, both animal and human studies have argued for a convergence of low threshold C fibre input to nociceptive fibre signalling at the dorsal horn level (3, 4, 7, 10, 14-17). On the basis of the behavioural observations of bilateral cold and tactile allodynia post unilateral median nerve injury (from *Paper I*) in this thesis, it is hypothesised that central interactions of fibres conducting noxious (i.e. nociceptors) as well as non-noxious stimuli (i.e.non-nociceptors) at the dorsal horn level are responsible in driving these sensations.

In addition, the actions of spinal immune cells in the dorsal horn such as microglia and astrocytes to influence nociceptive processing have been firmly established (18-22). Akin to the canonical perspective of attributing allodynia to A-LTMRs, the role of spinal glial cells in injury induced hypersensitivity has always been looked at in the context of these A-LTMRs in addition to the unmyelinated high threshold C nociceptors (20, 23, 24). From previous observations of such neuro-glial modulation mentioned above, it is hypothesized that the glial cells can modulate bilateral noxious signalling and the percept of allodynia evoked by otherwise innocuous stimuli, i.e. normally non-painful touch and cooling in this

model of bilateral allodynia due to a unilateral nerve injury. Incidentally, rapid cooling and non-painful gentle touch are hallmark of CTs in humans (5, 6) and in mice (10, 12, 13) and hence, cannot be ignored as one of the likely substrates of this phenomena in this rat model. In the dorsal horn, *lamina II*, which is the main termination site of CT inputs, has been postulated to act as a 'gate' for processing noxious as well as innocuous information by converging and processing inputs from superficial as well as deep dorsal horn (25). This work supports the view, also discussed in Abraira, Kuehn (26), that of low and high threshold inputs from the periphery, it is the dorsal horn (lamina I-III), that acts as the primary convergence centre to 'gate' or 'gain' painful and non-painful information before it is passed over to higher order neurons. Furthermore, by studying the neuronal and non-neuronal interactions in the dorsal horn at the peak time point of the bilateral allodynia seen in our model, it is possible to understand the biochemical changes responsible for this behaviour. These changes help us understand key mechanisms of protein signalling at the cellular dorsal horn that has been often overlooked in other experiments involving proteomics due to experimental bias.

Hence, the animal studies in this thesis provide a greater understanding of mechanisms of allodynia and its modulation using pharmacological tools in animals. In rats, following a unilateral median nerve injury, bilateral allodynia was ameliorated using minocycline, a glial inhibitor, by modulation of the dorsal horn interactions between glia and primary afferent fibres that respond to non-painful stimuli. These interactions (*Paper I & II*) were studied using immuno-staining and proteomic profiling of the dorsal horn – prior to and following nerve injury and its modulation by minocycline. The key result from the animal work, presented in this thesis, is the presentation of modulatory proteins responsible for the sensory behaviour at the contralateral, uninjured side as opposed to the primarily studied injured side of the dorsal horn.

To understand the 'gating' and 'gaining' mechanism of the dorsal horn discussed previously in rodent models, it is important to ask what dissimilates the perception of painfulness from non-painfulness or pleasure in humans with acute pain. Therefore, in healthy humans (*Paper III*), pain modulation was tested in the context of acute background muscle pain with concurrent 'affective' tactile stimulation. Furthermore, the resulting percept and its peripheral substrates was determined using a preferential block of

myelinated fibres. In this study, we demonstrated that activation of CTs can produce a stimulus-locked, bi-directional (excitatory/inhibitory) affective modulation of pain that is consistent with previously documented CT function in the rodent dorsal horn. From this human study, it is hypothesised that CT dependent context driven affective stimulus can determine the direction of the perception, in this case, pleasurable or painful. This study also presents with the idea that long term changes in the dorsal horn circuitry may not be necessary to initiate this type of context-dependent bi-directional stimulation and can be achieved in an acute setting.

Hence, the animal and human studies combine the animal sensory behaviour and human perception of allodynia (and its modulation) to explore the link between the activation of low threshold sensory fibres such as CTs (by innocuous stimuli) and nociceptive processing in acute and pathological pain states.

This thesis comprises of an introduction section and 3 manuscripts. The introduction provides a short review of the peripheral and central mechanisms of allodynia with the aim of interlacing it with the work presented in the 3 manuscripts (referred to in the text by their Roman numerals).

Papers included in the thesis

I. Shaikh S, Shortland P, Lauto A, Barton M, Morley JW, Mahns DA. Sensory perturbations using suture and sutureless repair of transected median nerve in rats. Somatosensory & motor research. 2016 Jan 2;33(1):20-8.

II. Implications of functional proteomics on the contralateral dorsal horn in the context of bilateral allodynia and glial changes post-injury. **Shaikh et al.** *(Under review in Pain journal)*

III. Shaikh S, Nagi SS, McGlone F, Mahns DA. Psychophysical investigations into the role of low-threshold C fibres in non-painful affective processing and pain modulation. PloS one. 2015 Sep 15;10(9):e0138299.

Introduction

1. Pain

By definition of the International Association for the Study of Pain (IASP), “pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. This sensation can result from highly complex organic, cellular or subcellular interactions, as a series, or as a part of other pathological changes in the body, integrated from the peripheral inputs via the dorsal horn to higher brain structures. Acute hypersensitivity has a highly valued protective purpose in response to injury and serves as a warning signal to prevent further damage. However, it is the chronic, long lasting hypersensitivity that is maladaptive as the symptoms often persist beyond repair of the initial lesion site (as shown in *Paper I*), which can be associated with long-term and often irreversible pathological and structural changes in the nervous system.

Classically, pain is elicited when specialised receptors in the periphery, called nociceptors, are activated, and the information is subsequently transmitted to the central nervous system (CNS) via dedicated high-fidelity pathways (*i.e.* labelled line model) (27-30). In addition to the primary afferent neurons, pro-inflammatory mediators, ATP, cytokines and growth factors, non-neuronal glial cells, and other immune-associated cells can have a major modulatory role in nociceptive processing (31-34). The primary afferents that encode the pain-eliciting stimulus from the periphery and relay the information to the spinal cord have their central terminals in the dorsal horn, which is the primary site of inhibitory or excitatory processing (25, 35, 36). A large number of pro-excitatory and pro-inhibitory interneurons reside in the dorsal horn that expresses several receptors which integrate signals and convey them to higher order structures via ascending modulatory pathways, which are then combined with the descending modulatory pathways from the brain to the dorsal horn that generate the overall sense of perception (6, 10, 13, 15, 17, 25, 37-41). The focus of this work is on the study of spinal bilateral dorsal horn in relation to the integration of excitatory and inhibitory processing by observing and quantifying the protein changes during and in absence of glial-allodynic modulation following an injury.

1.1. *Injury-induced hypersensitivity*

Hyperalgesia, which is an exaggerated painful response to a *noxious* stimulus, or allodynia, where pain arises from a normally *innocuous* stimulus (42), are two of the most studied pain symptoms. These sensations can be evoked reproducibly in experimental conditions using thermal, mechanical or chemical stimuli (2, 5, 6, 43). In humans, the capacity of subjects to describe the transition from non-painful to painful stimuli allows a distinction to be made between exaggerated responses to painful stimuli (hyperalgesia) and the emergence of painful responses to normally innocuous stimuli (allodynia), e.g. gentle touch or non-painful cooling (42).

1.2. *Afferents in nociception*

One of the earliest findings of cutaneous sensory fibres that mediate touch, pain and tickling sensations was in the 1930s (44, 45). 'Nociceptors' are identified based on their high threshold of activation and a capacity to encode high intensity (noxious) stimuli. There is a large amount of evidence that these fibres encode the intensity, duration and location of noxious stimuli along with signalling the quality of the painful sensation (28, 29, 46, 47). In addition, nociceptors can be sub-classified according to their response properties to preferred stimuli, degree of myelination (or lack thereof), conduction velocity and pattern of activity-dependent conduction slowing (especially for C fibres) (28, 30, 46-51).

As the majority of sensory behavioural testing in pain studies, including the work in this thesis, is focused on the skin, the focus of the discussion has been confined to cutaneous afferents. The cutaneous nociceptors can be thinly myelinated (A δ) or unmyelinated (C). Some C fibres respond to several types of noxious stimuli such as heat, mechanical pressure, cold and/or a variety of chemicals, and are thus called polymodal nociceptors. Other types of nociceptors may exhibit a degree of selectivity; for instance, they may respond only to noxious mechanical stimuli, or respond to noxious heat and mechanical stimuli but not noxious cold stimuli (29, 47, 50, 52, 53).

1.3. Peripheral vs centrally mediated nociception

Human microneurography (29, 50) and animal electrophysiology (52-54) experiments have shown that physiological stimulation of C nociceptors is associated with a response akin to noxious stimulation The area surrounding the site of injury, inflammation or trauma is considered a secondary zone of hypersensitivity. It was hypothesized that the perceived allodynia in the secondary zone was due to a spread of sensitization from the adjoining nociceptors from the area of the injury (2). That is, the activation of a nociceptor at the site of injury can lead to anti-dromic action potential propagation in other branches of the afferent resulting in the release of sensitizing substances (55, 56). This was explained using axonal reflex in the periphery and the release, as well as spread of, pro-nociceptive substances around the injured site that rendered hyper-excitability or sensitivity to non-injured nociceptors (55, 57, 58). However, several groups (59-61) have unsuccessfully tried to replicate these experiments in relation to the peripheral spread of pro-nociceptive signalling. This ambiguity, in part at least, arises when the origin of C nociceptive input and the region of allodynia are within the same circumscribed region of the skin (54, 62, 63), thus making it difficult to disentangle the allodynia that arises from peripheral sensitisation, from perturbed central integration of noxious and innocuous inputs. Other studies have demonstrated that, while an axon reflex has a peripheral origin, secondary hyperalgesia (and allodynia) is dependent on central – not peripheral – mechanisms (2, 64).

In acute pain states, it was shown that blocking the capsaicin-stimulated cutaneous site, did not reverse the secondary allodynia with pain persisting beyond the initial nociceptive drive (2, 51). Furthermore, by selectively stimulating large diameter fibres, Torebjörk's and colleagues (2) demonstrated the role of A-LTMRs in mediating mechanical hypersensitivity (allodynia), which was the result of perturbed central processing such that these low threshold A fibres were allowed access to the central nociceptive pathways.

Contrary to experimental models of acute pain, the mechanisms underpinning a chronic pain state may well be more complicated given the cascade of hyper-excitability events in the CNS that occur over a longer term (63, 65, 66), along with the phenotypic changes that might occur at the peripheral afferent level (see section 1.4 for more).

1.4. Peripheral fibre contributions to allodynia

Low threshold mechanoreceptors – myelinated (Aβ) or unmyelinated (C), Aβ-LTMR or C-LTMR respectively – encode non-painful tactile stimuli (67, 68). Human micro-stimulation studies by Torebjörk et al (2) demonstrated that selective activation of a low threshold Aβ fibres produced a tactile sensation in the absence of pain, but evoked an additional pain sensation (allodynia) following the induction of pain in an adjacent region of skin. This link between Aβ fibres and allodynia has been reinforced by the observation that the blockade of myelinated A fibres can abolish allodynia (2, 69-71).

In a series of human studies conducted by Nagi and Mahns (3-5, 7, 8), nerve compression was used to limit the afferent input to C fibres in order to test the role of CT fibres in tactile allodynia. In each case, the action of the block was confirmed by the abolition of tactile and cold sensibility whilst the preservation of C fibres was confirmed by the retention of warm sensibility. Under such conditions, the discriminatory aspect of touch (intensity, frequency and localisation) was abolished with any crude sense of touch being perceived as clearly innocuous. However, when hypertonic saline was infused into the underlying muscle (tibialis anterior), concurrent innocuous stimulation of the skin increased the overall pain ratings (i.e. allodynia). The anatomical separation between muscle (background pain/nociceptive input) and skin (tactile stimulation) compartments, supplied by a different branch of the peroneal nerve and independent vascular supplies, combined with a differential myelinated fibre block established earlier, meant that it was possible to systematically test the fibre class contributions to allodynia without any ambiguity as to whether it was generated by peripheral or central sensitisation. The same approach was adopted in *Paper III* of the thesis.

1.5. C-LTMRs: Animal equivalent of human CTs

Much like the human CTs (72), their functional equivalent in animals, called C-LTMRs, are also implicated in mechanical and cold allodynia in acute as well as in chronic pain states (10-13, 17). This class of mechanoreceptors (C-LTMRs) was first studied in cat skin (44) and since has been reported in several species including toads, rabbits and monkeys to name a few (73-78). However, these units were poorly studied in comparison to the high threshold C fibres or C nociceptors (28, 30, 49, 79) with questions about a clear, functional role and doubts about their presence in humans given the seemingly reduced density along the evolutionary timeline, i.e. from rodents to monkeys (53). Furthermore, in humans, tactile function has been almost exclusively examined through the prism of myelinated Aβ fibres (49, 80) even though the C-LTMRs are densely distributed in hairy skin and are found as frequently as their myelinated counterparts (30, 67, 81).

Unlike human experiments, perception cannot be readily examined in animals. Therefore, the contribution of C-LTMRs to gentle touch and cooling are inferred based on concordance of afferent responsiveness and behavioural responses. Such inferences have been most evident in several models of peripheral nerve injuries such as crush, constriction or ligation, and in inflammatory models using local application or injection of pro-nociceptive substances such as capsaicin or artemin. Also, since, it is known that partial nerve injuries in humans are more likely to develop allodynia than a complete nerve injury, several models used incomplete damage to the peripheral or the spinal nerve such as the spared nerve injury model (82)or the chronic constriction injury model (83) of the sciatic nerve and the spinal nerve ligation model (84) respectively. The injury model used in this work was a complete crush of the left median nerve sparing the radial and ulnar branches to develop a similar peripheral nerve model, but in the upper limb. In some studies combining these injury models with genetic modification in mice, the emergent hypersensitivities from glabrous skin of animal paws have been documented as behavioural evidence of cutaneous tactile and cold allodynia attributed to C-LTMR functionality (10-12) – a paradoxical observation that fails to recognise the ongoing ambiguity about the existence of this afferent class (or a functional equivalent of CTs) in glabrous skin (discussed in detail in section 5.4). Are these animal models comparable to human chronic pain pathologies? If so,

is the testing allodynia on the glabrous forepaws of these mice comparable to human perception of allodynia? Also, whether, the same class of fibres (C-LTMRs/CTs) can mediate allodynia in both species? As in many instances, human injuries involve complex and variable degree of damage as compared to a complete or uniform injury to a spinal or peripheral nerve, and hence such studies face several experimental limitations as opposed the frequent use of complete nerve injuries. Such limitations could include the varying degree of injuries, such as partial nerve damage that does not limit itself to a specific or whole part of a spinal segment, and hence, becomes difficult to test systematically. Another layer of ambiguity is added by the inconsistent, often patchy state of injured nerve in humans that is impossible to replicate or assess in animals. Since the advent of genetically modified mice, experiments can be conducted with a selective genetic expression or ablation in a group of animals, as a global or limited to a region of the body, as compared to naïve (wild type/WT) mice. For example, protein expression associated with a particular nerve fibre type or sensory transduction protein, e.g. transient receptor potential cation channel subfamily V member 1 (TRPV1) expressed on C nociceptors, can be studied in parallel to a nerve injury, something not yet achievable in humans.

In the last decade, researchers have found proteins such as vesicular glutamate transporters (VGLUT3 (11, 16)), voltage gated ion channels ($CaV_{3.2}$ (6, 12), $CaV_{3.3}$ (13)) and modulatory proteins (TAFA4, a chemokine-like protein that modulates injury induced allodynia (10) and GINIP, a GABA B-receptor modulator (15, 85)) to be preferentially expressed in unmyelinated low threshold mechano-receptors such as C-LTMRs. Additionally, transcriptional profiling of hairy skin C fibre endings has suggested distinct functional and genetic differences between the two non-peptidergic C fibres, namely, C-LTMRs that mediate innocuous touch and the $MRGPRD^{+}$, a marker of non-peptidergic mechano-nociceptors, free nerve endings responsible for mechanical nociception (13). Therefore, using RNA sequencing, immuno-labelling, sensory testing and electrophysiology in injured mice , it has become possible to label and study various afferent fibres in relation to touch, cooling, mechanical and cold allodynia as well as heat hyperalgesia, both in the periphery and in the dorsal horn circuits (10, 12, 13, 15, 37, 40, 86, 87).

2. Afferent convergence in the dorsal horn

In the periphery, primary afferent neuronal cell bodies reside in the dorsal root gangli (DRG) or trigeminal ganglia. The central terminals of these cell bodies terminate in the dorsal horn of the spinal cord where they interact with interneurons, other primary afferents, microglia and astrocytes (21). The dorsal horn has been segregated on the basis of the pattern of myelinated and unmyelinated fibre terminals that correspond to one or several sensory modalities (88). A simplified view suggests that the unmyelinated fibres project to the superficial dorsal horn (laminae I & II), while the myelinated fibres project to the deeper laminae of the dorsal horn (laminae III, IV, V & VI). More importantly, the superficial dorsal horn has a large number of unmyelinated high threshold C nociceptive inputs and conversely, the deeper lamina (such as laminae III) has myelinated low threshold Aβ input (11, 17, 25, 37, 40).

Neuronal circuits in the dorsal horn are complex and interconnected with dorsal horn neurons receiving synaptic inputs from both excitatory and inhibitory neurons. Recently, an extensive study in mice has been conducted on this circuitry that labels the organisation of low threshold mechanoreceptor (LTMR) region that processes information on tactile perception and allodynia (26). This data suggested that not only the LTMRs formed synapses with between four and eleven LTMR interneuron subtypes in the LTMR recipient zone (RZ) but also only a subset of LTMR projections convey 'processed' information to the post-synaptic ascending pathways to the brain. It was shown that the majority of this information processing was occurring in the dorsal horn region (in particular lamina II) and this region was the key to low threshold input modulation.

A separate study, in mice, investigated the functional organisation of the cutaneous low threshold mechano-receptive neuron terminals(LTMRs) in the dorsal horn as well as in the skin (88). In this study, neurons that mediate innocuous touch – classified into Aβ-, Aδ-, and C- LTMRs – were genetically labelled to reveal the pattern of axonal endings in hairy skin. This data analysis suggested that each hair follicle type is a functionally distinct mechanosensory end organ innervated by a unique and invariant combination of LTMRs. In particular, the C-LTMRs form longitudinal lanceolate endings associated with zigzag (80%)

and awl/auchene (20%) hair follicles that are TH^+ (tyrosine hydroxylase positive), $GFR\alpha2^+$ (GDNF family receptor positive) and $IB4^-$ (isolectin B4 negative) which are ~65% of the total cell number in mouse DRG and have distinct axonal terminals in lamina II_i of the dorsal horn. Therefore, concrete evidence exists for the functional organisation of LTMRs in spinal dorsal horn in mice that presents lamina II as a region central to innocuous mechanical transmission.

2.1. Excitatory and inhibitory lamina II transmission

Vesicular glutamate transporters, called *VGLUTs,* package glutamate into synaptic vesicles for release. In transgenic mice, $VGLUT3^+$ has been shown to co-localise with tyrosine hydroxylase (TH^+) in neurons that functionally identified as C-LTMRs and hence contribute to cutaneous allodynia observed following capsaicin-induced mechanical pain, inflammatory and nerve injury-induced pain (11, 17). These $VGLUT3^+$ TH^+ C-LTMRs terminate in lamina II_i (L2i), which receives low, not high, threshold nociceptive input (89, 90). Furthermore these terminals are co-localised with interneurons expressing PKCγ, which are also required for injury-induced mechanical hypersensitivity (90). Likewise, in oxaliplatin-induced neuropathy, in the same region of the dorsal horn, these $VGLUT3^+$ neurons were shown to play an important role in cold allodynia, another perturbed sensation mediated by C-LTMRs (6, 40).

In mice lacking VGLUT3 ($VGLUT3^{Cre}$), using C-Fos (a marker of neuronal activity), Pax2 (a marker for GABAergic neurons), PKCγ and calretinin labelling, multiple populations of excitatory interneurons were identified that could be associated with allodynia circuitry (25). Also, by using an anterograde trans-neuronal tracer, it was shown that transiently expressing $VGLUT3^+$ neurons in lamina III (Aβ-LTMRs) were presynaptic to other excitatory neurons, which were presynaptic to lamina II $VGLUT3^+$ neurons that co-localise with calretinin and mediate mechanical allodynia (25). Together, these low threshold inputs are required for polysynaptic transmission to lamina I neurones that process painful signalling.

In lamina II, calretinin, a calcium binding protein, is present in 85% of cells exhibiting large A-type potassium currents that receive strong excitatory synaptic input (91, 92). This excitability of neurons in lamina II can be due to phosphorylation of Kv4.2, a downstream

target for ERK, a marker for noxious stimulation in many neurons (primarily in superficial dorsal horn) and mitogen activated kinase (MAPK) pathway, that activates microglial cells (93, 94). However, in the same region cells displayed low threshold T-type calcium currents typical of the cells receiving weak excitatory input. Furthermore, cells in this region receives input from both excitatory (strong and week) as well as Pax2 expressing GABAergic inhibitory interneurons (91). Furthermore, human findings in relation to this calcium channel reinforces the role of CTs in detection of innocuous touch and cooling that can evoke allodynia in the context of background nociceptive activity suggesting an intricate dorsal horn circuitry in place responsible for processing excitatory and inhibitory inputs (6, 8).

Supporting this view, a study on dorsal horn circuitry suggests that in order to evoke allodynia, Aβ-LTMRs evoke a disinhibition of projection neurons in lamina I, i.e. $NK1R^{+}$, a neurokinin 1 receptor, a class of G protein coupled receptors, via pre-existing polysynaptic connections (38, 41, 95) (*see figure 1*). Similarly, there are inhibitory circuits providing disinhibition to lamina I nociceptors emerging from lamina II_i C-LTMRs that express a GABA inhibitory protein (GINIP) (15, 85), which counter balances excitatory transmission from the VGLUTs. Subsequent knock out (KO) of TAFA4 and GINIP ($G\alpha_i$-interacting protein) in mice revealed inhibitory signalling in lamina II was not limited to chronic inflammatory or nerve injury-induced allodynia but could also be attributed to acute pain processing (10, 15), suggesting that functionally limiting C-LTMR-mediated inhibition can contribute to the development of chronic pain. This GINIP-mediated GABAergic signalling receptors are found in excitatory and inhibitory synapses in the superficial dorsal horn and at the primary afferent terminals (39). Therefore, while TAFA4 can produce hyper-excitability associated with C-LTMRs, both TAFA4 and GINIP can also do the reversal and end painful symptoms, thus making them potential therapeutic targets.

In contrast to the opposing roles of modulatory proteins (e.g. TAFA4, VGLUT3 and GINIP) expressed by C-LTMRs in the spinal cord of mice, the use of a T-type calcium channel antagonist in the periphery that blocks $CaV_{3.2}$ and $CaV_{3.3}$ abolished experimentally evoked cold allodynia (6) and diminished tactile sensitivity (8) acutely in humans. Notably, the T-type calcium currents include the $CaV_{3.2}$ and the $CaV_{3.3}$ subset, associated with mechanical and cold allodynia that are preferentially expressed on C-LTMRs in mice (12, 13, 85).

Consistent with the findings in humans, functional analysis of $CaV_{3.2}$ channel expressed on mouse C-LTMRs revealed their responsiveness to cooling and increase in mechanical thresholds during its inhibition (12). Consistent with this demonstration knockout of $CaV_{3.2}$ channel in injured mice suppressed the perception of innocuous touch, tactile and cold allodynia (12), findings that have been replicated in human reports of CT functionality (6, 8). Furthermore, RNA sequencing data showed $CaV_{3.3}$ channel to be specific to C-LTMRs and distinct from polymodal C nociceptors (13).

With this information, it is important to understand the dorsal horn architecture and circuitry in place for processing of innocuous (tactile/cold) and noxious inputs, i.e. integration in lamina II. Moreover, it is important to acknowledge that this circuitry works conjointly to process innocuous and noxious stimuli, instead of attributing a lone sensory modality or sub-modality to the narrow limits of a single class of afferent (26). With its extensive inhibitory and excitatory networks, this region of the spinal cord acts as a 'gate' that normally segregates innocuous myelinated inputs (laminae III-IV) from nociceptive processing in superficial layers (and projection neurons in lamina I). The lamina II region, with extensive projections from C-LTMRs, is equipped with all the microcircuitry (see figure 1) required for the superficial dorsal horn to evoke allodynia without the need for elaborate anatomical reorganisation (discussed further in the following section) (96). Human models of acute muscle pain have shown that CT-mediated allodynia can be evoked acutely (within seconds) using vibration and gentle brushing, hence the induction of allodynia seems unlikely to depend on structural reorganisation at the spinal level (3-5).

2.2. Of spines and sprouts: Is allodynia dependent on the anatomical reorganisation of the dorsal horn?

Classically, cutaneous allodynia has been attributed to myelinated A-LTMRs (1, 2, 62, 97). In many studies, this link was characterised using a capsaicin model where a circumscribed allodynia in the skin (2) was dependent on ongoing pain (69, 70, 98, 99). These Aβ fibres, which are normally implicated in low threshold mechano-sensation, do not evoke pain responses under normal conditions. There is a popular argument that following a peripheral nerve injury, the Aβ-LTMRs which are known to project from the deeper laminae

of the dorsal horn (III & IV), to the superficial laminae to establish functional contacts with nociceptive pathways to evoke cutaneous allodynia (100-103). This view suggests that a system must survive an injury in order to gain functional connectivity within the areas implicated in pain processing.

Under diseased conditions such as inflammation and injury, it was hypothesised that a 'phenotypic switch' occurs within this class of A fibres as they synthesize substance P (SP), a pain signalling protein that is normally restricted to nociceptors (96, 104). It has also been show that this SP can be released following electrical stimulation of Aβ fibres in injured rats with spinal nerve ligation (105). In contrast, others (106) have shown that SP is not released from injured Aβ-LTMR and do not contribute to allodynia in these rats.

In contrast to the 'canonical' view of Aβ-mediated allodynia, convergent evidence from psychophysical (3-5) and microneurography studies, has revealed that cutaneous allodynia can persist following the blockade of myelinated fibres, suggesting a role for unmyelinated fibres in this phenomenon. As allodynia can be evoked in a matter of seconds in acute pain states, this suggests that an elaborate central reorganisation or long term sensitisation of primary afferent terminals need not be required to generate this effect (3-5). Not only does this observation question the need for structural changes for *initiation* of allodynia, it also argues against the purported exclusivity of Aβ mechano-receptors in generating allodynia (3-5). However, the "quantitatively small" level of spinal reorganisation need not exclude it as a contributing factor to the *persistence* of allodynia (107). In addition to Aβ mechano-receptors (A-LTMRs), there exists a separate class of Aβ high-threshold mechanoreceptors (A-HTMRs) in non-human species including monkeys (108), which, while normally brush-insensitive (i.e. high-threshold), have been hypothesised to contribute to pain hypersensitivity (punctate / brush allodynia) following a drop in thresholds post-injury or inflammation.

Early work (109) using the B subunit of cholera toxin (CTb) as a marker of Aβ-LTMRs suggested that after a peripheral nerve injury, Aβ-LTMRs sprout in the superficial dorsal horn (laminae I and II) whereas in the absence of an injury, CTb was not found in lamina II_o (outer). This suggested that central sprouting had contributed to CTb labelling following injury, however subsequent studies have raised concerns about his interpretation given the

subsequent demonstration that CTb uptake and labels injured C fibres in lamina II_o (110, 111). In addition, it was shown that the pattern of intra axonal labelling did not differ after injury (96, 112) with marked similarities between myelinated nociceptors and LTMR axonal arbours that extend into lamina I in uninjured animals (96). The potential confound that both fibres classes can take up CTb may well account for the assertion that Aβ fibres extending into lamina I and II following injury (109).

Although, there is an association between injury and inflammation states that increases in polysynaptic responses to of neurones in the superficial laminae to inputs from Aβ fibres (95, 113, 114), recent findings have suggested a complex circuitry of LTMR (both A- and C- subtypes) excitatory and inhibitory interneurons receiving direct synaptic inputs from multiple LTMR subtypes at any given point without the need for an injury or inflammation (26). Data from this study suggests a parallel LTMR module within the dorsal horn architecture that integrates low threshold information based on the context specific cues from the periphery (i.e. pain, injury and inflammation) thereby selectively gating one or more modalities given a change in a physiological state (26). Hence, allodynia need not be dependent on the projection of only large diameter inputs into the superficial lamina of the dorsal horn and ***can*** be mediated by the low threshold C afferents in both humans and animals. Whereas in the normal state that the overall perception is one of tactile sensibility with the affective (pleasure vis-à-vis unpleasant) being determined by the balance of parallel processing of A and C LTMR subtypes. Therefore, it becomes imperative that allodynia is studied in the context of dorsal horn architecture instead of relying alone on a lone archaic view, i.e. A fibre mediated allodynia.

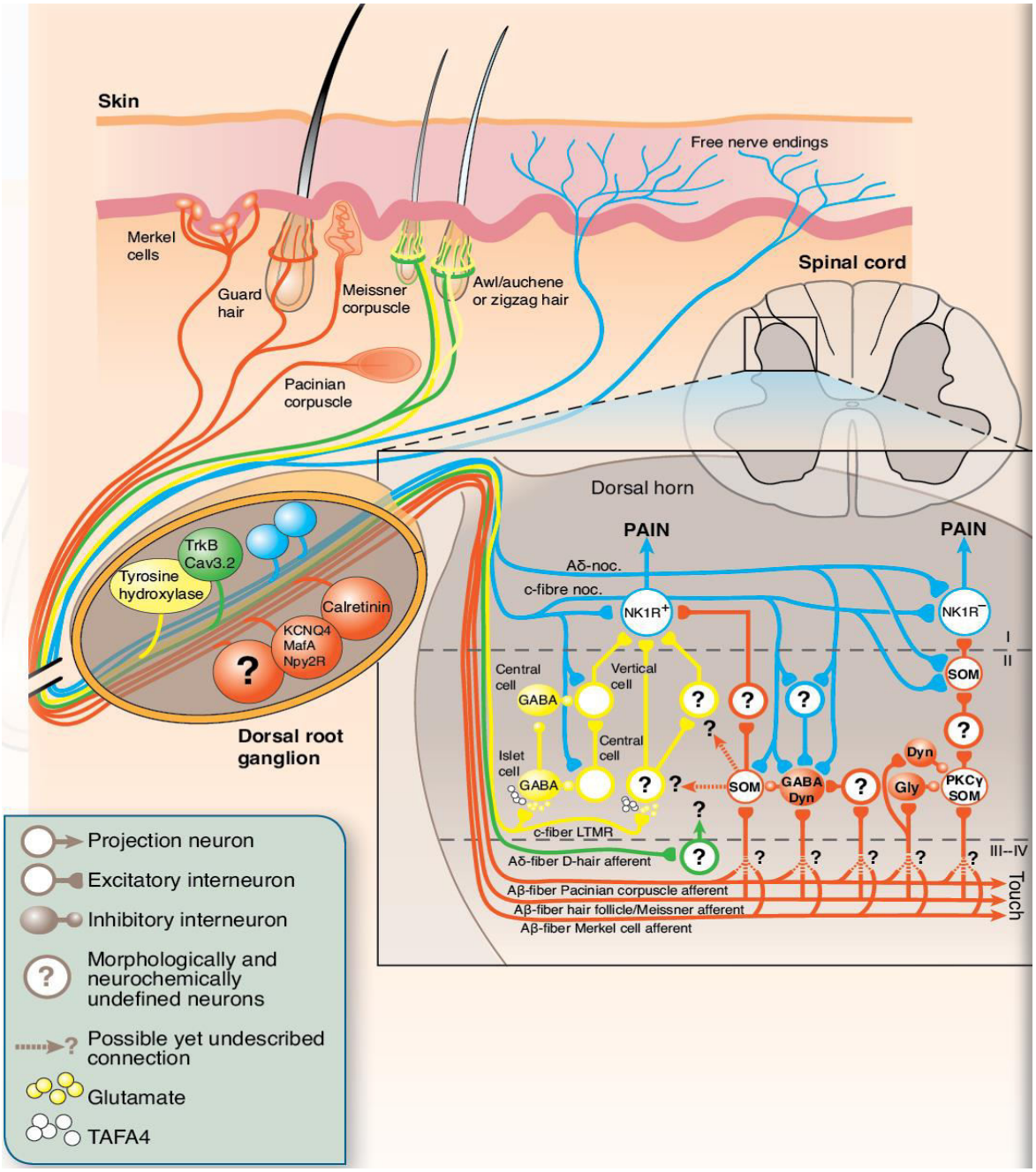

Figure 1 *Spinal dorsal horn circuitry adapted from Arcourt and Lechner (37), Lawton and Pimm (102)*

3. The significance of contralateral effects

Since, in our first animal model of neuropathic pain, i.e. a nerve transection and repair using chitosan, a unilateral nerve injury that induced bilateral tactile and cold allodynia which was traced over a 90 day period (Paper I). This established our model and defined timelines that suggested that first 10 day period following the nerve injury were critical to the development of sensory perturbations following injury. The results showed us that animals were at their lowest sensory thresholds for cold and tactile allodynia as well as heat hyperalgesia during that initial period. As our aim in this thesis was to identify the molecular basis of the change in sensory function, rather than the efficacy of the repair technique, we elected to test whether a similar hypersensitivity was observed following a median nerve crush in the forearm. This allowed us to assess sensory function prior to- and immediately following nerve injury. This also allowed us to study whether these bilateral effects were limited to a particular form of nerve repair, such as chitosan, or can be extended to other forms of nerve injury such as crush. Also, unlike other models such as a partial nerve injury, nerve crush produced a complete damage to afferents leaving only the translucent epineurium intact. In addition to that, the previously used spared nerve injury models (82, 115) were used as a lower limb model of injury. Our study (Paper I & II), is the first instance of an upper limb model that showed the bilateral effects of cold and tactile hypersensitivity following a unilateral median nerve injury.

M

4. Glia and allodynia

Outnumbering the neurones within the complex spinal circuits are the immune support cells, in particular, microglia and astrocytes, which normally stabilise and buffer ion and neurotransmitter concentrations. These glial cells also undergo marked changes in morphology and expression following peripheral injury or local inflammation (19-21, 31).

Like any other areas of dorsal horn, lamina II contains an extensive network of glia support cells. The fact that in our animal experimental model (*Paper I*), the tactile and cold allodynia observed following peripheral nerve injury extended bilaterally suggests a central involvement mechanism reliant on the integration of neural activity supported by glial cells. In order to test whether the emergence of cutaneous allodynia is dependent on glial activation, a glial modulatory drug such, minocycline, was used as a tool to study these interactions at the dorsal horn level. In animal work, minocycline has been used in models of chronic neuropathic and inflammatory pain (115, 116). Broadly, minocycline, a weak tetracycline derivative, is known for its neuroprotective qualities by inhibition of active and proliferating microglia (117). It has been studied in various mice models of conditions that cause inflammation such as Parkinson's, amyotrophic lateral sclerosis and Huntington's disease (118-120). In humans, while familiar in the context of treating skin conditions such as acne, it has been recently used in clinical trials for chronic neuropathic pain (121-123) as well as experimental pain (9) for the treatment of cold and tactile allodynia.

It was hypothesized that following peripheral nerve injury, glial activation, or gliosis induced localised inflammation that was supported by changes in enzyme expression and increased energy consumption. Studies including ours demonstrate the delivery of glucose to neurons and glia by the increased expression of glucose transporter proteins in this state (124, 125). Such release of stored glucose, use of ATP and increase in expression of enzymes of the glycolytic pathway create the environment that exposes cells to oxidative stress in which makes glial cells take on a protective role in neuropathies (126). Moreover, the ATP released from activated astrocytes can inhibit GABAergic inhibitory interneurons, via A1-receptor-dependent pathway, to produce hypersensitivity (127). Therefore, it can be said that glial activation contributes to the cellular stress observed in injury induced

hypersensitivities and consequently modulating this glia response can have an impact on painful processing including the emergence of allodynia.

Hence, as shown in paper II, we tested whether minocycline could modulate the ***a.*** bilateral allodynia in the upper limb in injured animals as compared to non-injured animals and ***b.*** bilateral changes in glial cell morphology and expression in the lamina II region that correlated with allodynic behaviour and ***c.*** bilateral spinal dorsal horn protein changes responsible for the overall pain processing in glial as well as in neuronal tissues.

Since, the emergence of bilateral allodynia questioned the use of contralateral side as control as opposed to naïve (non-injured) animals, correlating minocycline driven bilateral glial changes in injured and naïve animals, with behavioural tests of tactile and cold allodynia, painful processing was confirmed to be dependent on neuro-glial interactions. In the design of the experiment it was hypothesised that studying the protein expression in the dorsal horn with concomitant minocycline pre-treatment can uncover important details of these glial and neuronal interactions prior to and during disinhibition of allodynic processing, knowing that C-LTMRs in lamina II, along with fibres in lamina I and II, are part of this 'connectome' that drives injury-induced hypersensitivity. More importantly, the different protein species (i.e. isoforms and post-translationally modified variants) observed on the contralateral side in an injured system can reveal changes that are generally masked due to the dominance of local proteins related to the structural disintegration and inflammation on the injured side. Hence, an **unbiased blinded approach** to analyse the protein dataset can reveal the otherwise hidden important proteins related to initiation or maintenance of allodynia, which can be seen contra-laterally, and are largely lost during shotgun sequencing. Therefore, in the proteomic results (*Paper II*), we expected to find several structural, metabolic, growth, signalling, folding and chaperone proteins which can be modulated using minocycline which also reflected in their behaviour and glial morphology.

In our human model of acute muscle pain, the intensity of pain was found to be modulated by concurrent affective-tactile stimulation (*see Paper III for more*). Indeed, the contribution of CTs to the affective dimension of somatic sensations has been shown previously, with CT input projecting to various regions of the limbic as well as the paralimbic

system such as the insular cortex (128, 129). In the light of convergent information, we hypothesised interactions between peripheral LTMR input (such as C-LTMRs) and glial cells, and tested the role of minocycline in modulating these interactions by gauging changes to pain hypersensitivity state, namely, to innocuous mechanical and cold stimuli. A human work minocycline pre-treatment with minocycline abolished the emergence of bilateral cutaneous cold allodynia and the emergence of bilateral muscle tenderness following repeated injections of Hypertonic saline into one leg (130). Although previous animal and human studies have linked allodynia with C-LTMRs and CTs, our animal studies cannot definitively determine that these interaction are only driven by these two afferent populations (4, 6, 8, 10-12). Indeed, other low threshold afferents (e.g. large diameter LTMRs) mediate the hypersensitivities found in these models along with other high threshold afferents (HTMRs) that mediate unilateral heat hypersensitivities. As discussed earlier, these LTMR interactions are mainly occurring in parallel in the dorsal horn, the focus of this work has been limited to studying the interactions of glia and sensory afferent, interneuron populations by studying protein interactions within the dorsal horn region.

5. CT-mediated function in humans

5.1. C fibre-mediated allodynia

Psychophysical studies, conducted by Nagi and Mahns (3-5, 8), showed that allodynia can be mediated independently of myelinated fibres via intact CTs in humans during acute muscle pain. Under a myelinated fibre block, the allodynia evoked by otherwise non-painful tactile stimuli, such as slow brush strokes (4, 5, 81), was indistinguishable from the all-fibre-intact state, but was blocked using a low concentration of local anaesthetic targeted to block C fibres. As documented earlier (131, 132) and in the above mentioned studies, central to the criteria for assessing the efficacy of compression block was that, as the compression progressed, the Aβ mediated detection of vibration and Aδ mediated cold sensations were lost, while C-fibre mediated warmth remained intact. This use of compression block permitted a short window of time (~20 min) to test whether affective touch (brushing) stimuli or focal vibration before the compression block progressed to C fibres inputs. This timeframe and progression rate is known to vary from site to site (133, 134) and from person to person depending in a manner that is not readily predicted by subcutaneous tissue and the depth of the nerve, hence necessitating the functional confirmation of the extent of blockade in each subject prior to the application of test stimuli. The contribution of CTs to pain processing was not due to disinhibition arising from myelinated blockade alone, as the allodynia was not observed when test stimuli were delivered following compression block in the absence of pain. In contrast the allodynia observed during ongoing pain was abolished when the C fibres were preferentially blocked (with the myelinated fibres intact).. In a recent study using microneurography (135), it was noted that CTs in humans follow high frequency stimulation. In paper III, we built on earlier observations that compression block, while abolishing the discriminative aspects of touch the perception of affective aspects touch (i.e., pleasure or unpleasant) of vibration remained intact. Non-painful focal vibration of varying frequencies applied during myelinated fibre block (5) resulted in affective responses typical of those attributed to CT activation and produced a context-dependent manner to modulate pain intensity (*see Paper III for more*). Early work showed that non-noxious cooling of the skin can activate CTs (67) –

an observation which was then replicated in the context of allodynia following a myelinated fibre block (6).

Interestingly, in healthy participants, using hypertonic saline-induced acute muscle pain in tibialis anterior muscle (TA) and stimulus activation paradigms for CTs, such as innocuous touch and dynamic cooling, applied concurrently on the skin overlying TA, it was shown that the allodynia persisted following myelinated fibre block, despite the anatomical compartmentalisation of skin and muscle. Moreover, allodynia persisted even when the separation between the site of pain induction – the flexor carpi ulnaris (FCU) muscle (innervated by the ulnar nerve) – and the site of innocuous stimulation was increased by moving from the hairy skin of the forearm (supplied by the radial nerve), to the distal glabrous skin of the little finger (supplied by of the ulnar nerve) and ultimately, to the index finger (median nerve) (3). Also, there is preliminary data that allodynia is observed contra-laterally when muscle pain is induced in one leg (9). This anatomical separation between the site of noxious and innocuous stimulation ensures that any allodynia observed is the consequence of a perturbed central integration of sensory inputs rather than a change in primary afferent responsiveness or peripheral sensitisation. The generalized expression of CT-mediated experimental allodynia is unique given earlier reports of a strictly circumscribed expression of allodynia, which was attributed to Aβ-LTMRs (2).

In addition to allowing a range of mechanical, chemical and thermal stimuli to be applied to the skin, with ongoing/background pain in the muscle, this model also allows localised intradermal application of pharmacological agents that specifically block CTs (8). With the use of new pharmacological agents, it has been shown that CT-mediated cold allodynia can be abolished using CT antagonist (T-type $CaV_{3.2}$ channel blocker, TTA-A2) in human skin (6). Likewise, CT-mediated touch has also been abolished using the same antagonist in human palmar skin (8), where electrophysiological evidence for the existence of human CTs is lacking (discussed further in section 5.4). While the majority of animal behavioural tests (including those for CTs) are conducted in the glabrous skin of the paws, the narrative around CT-mediated sensations remains confined to the hairy skin (10, 11, 17).

5.2. Pain and affect in relation to CTs

Pain comprises of a multidimensional sensory experience that includes affective (emotional) and discriminatory aspects. In the last century, the focus was mostly on the discriminatory aspect of pain, such as intensity, location and quality. In the last two decades we have begun to understand the substrates subserving the affective component of pain (136, 137).

Pain is produced by stimulation of high threshold nociceptive C fibres, polymodal free nerve endings sensitive to noxious temperatures, chemicals such as capsaicin, exposure to pro-inflammatory mediators and noxious mechanical pressure that can potentially cause tissue damage. Conversely, affective sensations have been attributed to activation of a special class of low threshold C fibres that responds to slow, gentle mechanical stimulation: CT afferents, in the context of touch (81, 138-141) as well as pain (4, 5, 8, 14). These are particularly sensitive to slow stroking across the skin such as brushing, which is normally perceived as pleasant.

A combination of psychophysics and imaging (fMRI) studies have demonstrated the role of CTs in pleasant touch in humans (81, 128, 138). In particular, a study in large fibre de-afferented patients (n=2) has shown that diffuse and weak pleasant sensations can be evoked using gentle brush stroking known to activate the CT afferents (128). However, in these patients, the thermo-sensory (including warmth) thresholds were reported outside the normal range of detection, thus the impairment may not be limited to the myelinated system (140, 141). Furthermore, the sensations were inconsistent from trial to trial and required a 2-alternative forced choice paradigm to reveal any contribution to perception (81).

In healthy subjects, CT activation, using graded brushing velocities, revealed an 'inverted U-shaped response' (negative quadratic) with peak neural responses occurring at a moderate velocity range of 1-10 cm/s, which was perceived as most pleasant, and slower or faster velocities perceived as unpleasant. The observations in the two de-afferented patients [79] and the demonstration of an inverted U-shaped stimulus-response relationship for CT discharge formed the basis of the CT-pleasant touch hypothesis.

Intriguingly, CT afferents also fire at slow velocity brushing when subjects report neutral or negative affective, i.e. unpleasant, sensations, indicated below the neutral line in

the inverted U-shaped curve (81). Careful inspection of the data reveals that the same brushing stimuli evoked discharge in myelinated and unmyelinated fibres, with the sense of pleasantness emerging at the point where the discharge of myelinated afferent fibres exceeds that of CTs.

5.3. Dual modality of CTs

The perceptual line separating pleasant touch and pain is not static, but can be reconfigured by varying forms of acute or chronic muscle pain. The experiments with CT stimulation shown in this thesis (*see Paper III for more*) can produce allodynia. In other human studies, repeated episodes of muscle pain, produced by physical activity (4) or acute intramuscular injection of hypertonic saline, superimposed with gentle touch (3, 5) and innocuous cooling (6) were perceived as painful – a phenotype consistently linked to the activation of CT afferents. These experiments suggest that tactile and cold allodynia arise when normal sensory traffic is misinterpreted as painful. Interestingly, tactile and cold allodynia accounts for over 70% of the variability in symptoms reported by patients with neuropathic pain (142, 143).

The role of CTs in pain processing is seemingly contrary to the pleasant touch hypothesis. These observations suggest that either CTs have a dual modality (determined by the evoked temporal patterns) or another class of CT afferent is modulating pain responses, different from the CTs encoding pleasant touch. As shown in *Paper III*, a context-specific contribution of CTs could modulate pain based on the positive or negative affective attributes of the stimulus. Moreover, this idea is also supported by studies in mice dorsal horn circuitry (26) that suggests computations of context specific and selective gating of modality under a specific physiological state, such as acute muscle pain in humans, which may be responsible for affective modulation of allodynia shown in humans.

5.4. Comparison across skin types

Human microneurography studies have not found CTs in glabrous skin yet. However, with a few exceptions of human C nociceptor recordings in glabrous skin (30), the majority of the C-fibre recordings in general have been carried out in hairy skin (6, 81, 139, 140, 144).

Recently, a functionally equivalent class of low threshold C fibres in human glabrous skin was reported that was functionally similar to its hairy-skin counterpart (3). Using psychophysics, it was demonstrated that this class of CTs also mediated mechanical allodynia in acute muscle pain. Furthermore, Nagi, Dunn (8) showed that detection of low threshold von Frey filaments (in the condition of blocked myelinated afferents) – a stimulus known to activate CTs – can be blocked using the same $CaV_{3.2}$ channel antagonist (TTA-A2) that was used and confirmed to block CT-mediated cold allodynia in humans by Samour, Nagi (6). In rats, recent C fibre recordings revealed tactile and cooling responses consistent with C-LTMRs, the animal counterpart of human CTs, which indicates the existence of these fibres in the rat glabrous skin (86) and replicated the earlier recordings from lamina I spinal neurons (145).

The majority of behavioural tests conducted in animal neuropathic pain models for mechanical as well as cold allodynia (including the work presented in *Paper I* & *II*) are carried out in the glabrous skin, but there is little focus on the skin region tested for such experiments. Therefore, although there is an absence of histological evidence for C-LTMRs in glabrous skin, there is convergent animal and human evidence to suggest a class of low-threshold C fibres in glabrous skin that is functionally equivalent to CTs or C-LTMRs in hairy skin – an argument that had been previously entertained for their large diameter counterparts in hairy and glabrous skin, e.g. hair follicle afferents and Meissner corpuscles (146-148).

Aims of the thesis

1. To establish an animal model of chronic nerve injury-induced pain in order to reliably test tactile and cold allodynia using established and novel methods of sensory testing and monitor nerve recovery following median nerve injury (*Paper I*)

2. To study the effect of minocycline, a microglial inhibitor on bilateral allodynia and (*Paper II*)
    a. In the same group of animals, compare allodynic behaviour with dorsal horn lamina II glial expression and morphology in treated & naïve animals(*Paper II*)
    b. In the same group of animals, using unbiased proteomic profiling, analyse regulation of protein expression in the injured dorsal horn and after the administration of minocycline in ipsi- and contra-lateral dorsal horn (*Paper II*)

3. To study the involvement of human CTs in processing of non-painful affective-touch (unpleasantness and pleasantness) and pain (allodynia and analgesia) using psychophysics in healthy subjects (*Paper III*).

Overview of the Papers

Paper I: Rodent models of long lasting bilateral tactile and cold allodynia

In the first study, we tested the sensory behaviour of rodents that underwent surgery for suture-less median nerve repair technique (149) established earlier in our lab. This study established an animal model of chronic pain based on unilateral median nerve injury and determined a long term recovery timeline using innocuous and noxious stimuli. Although, the pragmatic aim of our experiments was to develop the repair technique, the demonstration of prolonged tactile and cold allodynia provided a new paradigm to assess sensory behaviour.

One week following median nerve injury at the site of the axilla, we showed:

- Bilateral cold and tactile allodynia
- Unilateral heat hyperalgesia
- Return of sensation in the forepaw within 10 days – nerve injury under the arm
- Hypersensitivity outlasted the initial site of injury
- Significant but relatively short-lived bilateral hypersensitivity observed following a sham operation.

Based on these observations, we decided to study the mechanisms of marked bilateral allodynia and its rapid onset, i.e. before the nerve regenerated in the glabrous skin. It was hypothesised that these sensory perturbations involved a central processing at a dorsal horn level that included both neural and non-neural (glial) changes.

Paper II: Reversal of bilateral allodynia with glial inhibition and proteomic profiling of key changes in the dorsal horn

To test the involvement of glial cell activation in tactile and cold allodynia observed in study I, we used minocycline, known to suppress microglial activation (18, 150), immediately post peripheral nerve crush to observe whether this attenuated the glial cell responses to nerve injury and the emergence of cutaneous allodynia. Following nerve injury, rodents were sacrificed at the peak of cutaneous allodynia, at day 8 (i.e. at their lowest tactile, cooling and highest heat thresholds), and antibodies against microglia (IBA1) and astrocytes (GFAP) were used to quantify the change in the ipsi- and contra-lateral dorsal horn (DH) of the spinal cord. Quantification was focused on the area in DH lamina II_i, an area known to gate innocuous and noxious inputs.

A recent study showed that microglia are not required for mechanical pain hypersensitivity in female mice and used adaptive immune cells, likely T lymphocytes instead (151). However, in our species of rats, there was no difference in behaviour for pain sensitivity between males and females and hence, the distinction in gender was not made while allocating these rats, i.e. at any given time, there were both males and females in one group.

Secondly, to study the proteomic changes in rodents with allodynia, blinded 2D gel electrophoresis (2D- SDS PAGE) and mass spectrometry was conducted on the ipsi- and contra-lateral dorsal horn of the injured minocycline-treated animals and compared with control animals. Behavioural and histological assessments were repeated in control and minocycline pre-treated animals

In injured animals, minocycline:

- Prevented the development of bilateral tactile and cold allodynia
- Inhibited the bilateral microglial activity measured with $IBA1^+$ microglial cells
- Modulated the bilateral astrocytic activity measured with $GFAP^+$ astrocytes
- Suppressed protein expression in the dorsal horn that may be involved in the development and/or maintenance of neuropathic pain behaviour.

- Using dorsal horn of naïve animals, as opposed to the contra-lateral side, revealed a significant proportion of important proteins that would be otherwise hidden by the structural changes on the dominant ipsi-lateral side

By using minocycline pre-treatment to modulate bilateral allodynia post-unilateral median nerve injury, only those proteins associated with the change in condition were analysed as a function of change in behaviour. Glial associated changes on both sides of the dorsal horn were also observed to confirm the surgery and the impact of minocycline modulated behaviour. Since minocycline is a microglial inhibitor, as expected, the sensory behaviour correlated with microglial activation and proliferation. Furthermore, this cascade of inhibition also affected the astrocyte activity. As discussed earlier, allodynia can be mediated by A- and C-fibres (5, 6, 8, 25), these glial changes were specifically quantified in the lamina IIi as the region is known to have key importance in C-fibre mediating allodynia (11, 25). Furthermore, the direction of change in proteins observed bilaterally as a function of sensory modulation due to minocycline implies these proteins may have a key role in bilateral hypersensitivity (i.e. tactile and cold allodynia).

The results suggest that behaviour, glial activation-deactivation and protein changes associated unilateral injury is not limited to the ipsilateral side but extends to the contralateral side. Consequently, the contralateral side cannot be regarded a reliable control. Is this the key to observing small but functionally important (protein) changes for understanding the central mechanisms of allodynia? Furthermore, minocycline inhibits a number of proteins that may be responsible functionally for pain behaviour in these animals.

These observations also provide information on dorsal horn neuronal and neuro-glial interactions in different pathological states, i.e. glial inhibition, activation and following nerve injury. This study has important implications for the development of novel protein targets for pain treatment such as information about novel receptors, molecules and their pattern of expression in relation to injury-induced allodynia.

Paper III: CTs in a bimodal effect – allodynia and analgesia

Since allodynia, a major symptom of chronic pain, was the focus of our animal work, a parallel human study was conducted to test the afferent fibre contributions to pain and affect during acute pain, with emphasis on the contribution of CTs that are known to mediate allodynia (3-6) and affective touch (14). In this study, we tested two seemingly divergent theories, i.e. CT-mediated pleasantness in non-painful conditions and CT-mediated allodynia during background pain or nociceptive input in healthy human participants using intra-muscular hypertonic saline infusion.

We found that the application of previously pleasant (positive affect; velvet) stimuli to the skin overlying the painful muscle resulted in overall pain relief, whereas the application of previously unpleasant (negative affect; sand paper) stimuli resulted in overall pain intensification. These effects were evoked regardless of whether the myelinated fibres were intact or not. This demonstrates that both positive and negative affect, and context-dependent analgesia and allodynia, can be subserved by a low-threshold unmyelinated substrate, i.e. CTs.

At the peripheral level, this alludes to the role of temporal coding in affective processing or possibly the existence of undefined subtypes within the C-LTMR class which warrants further investigations. At the spinal level, the interplay between these bimodal processes vis-à-vis C-LTMR inputs could be explained by the excitatory and inhibitory proteins and molecules such as VGLUT3 and TAFA4 in the dorsal horn area of C-LTMR projections reported in mice. Therefore, the results from this study, and the animal studies of allodynia, contribute to our understanding of this region of the spinal cord and, by implication, modulation of pain processes.

Conclusion

This thesis establishes that:

Paper I: A novel rodent model of unilateral upper limb nerve injury that produced bilateral allodynia and its recovery timeline using sensory behaviour. These behavioural tests included testing for heat hyperalgesia, tactile and cold allodynia that prolonged, bilaterally, long after the initial site of lesion was healed. Moreover, a new method to test generalised cold allodynia was established.

Paper II: Akin to the model in Paper I, nerve injury with a shorter recovery time, minocycline-treated (i.p.) animals failed to develop bilateral allodynia when compared to normal saline-treated control. While immuno-labelling confirmed bilateral activation of microglia and astrocytes in injured animals, this effect was reversed using minocycline, a microglial inhibitor, that not only supressed microglial activity but also modulated astrocytes. Furthermore, proteomic changes in the dorsal horn, bilaterally, revealed several important proteins associated with glial function that were supressed with minocycline. Some of the top proteins measured on the contra-lateral side revealed small but significant changes that remained hidden on the ipsi-lateral side and were distinct from the naïve groups. This effect could be attributed to the major structural proteins and its breakdown dominating on the injured side, some of them functionally unrelated to the heightened nociceptive function. Several of these proteins have no recognised function corresponding to pain pathologies. Nevertheless, these proteins represent the biochemical milieu, in the dorsal horn, during those events of sensory changes. More importantly, it also questions the affiliation of a single pain signalling protein or its gene expression to vast behavioural symptoms in pain and its complicated system interactions. Therefore, injury-induced bilateral allodynia, and its inhibition using minocycline, was observed functionally using behaviour testing, in cellular morphology within dorsal horn as well as in the molecular and proteomic changes in the dorsal horn.

Paper III: Finally, the results from this Paper reconcile two seemingly opposing observations, i.e. CT-mediated pleasant touch in the normal condition and tactile allodynia during

background pain. We demonstrate that low threshold C-fibre input drives allodynia and analgesia in a context-dependent manner where the application of pleasant and unpleasant (non-painful) stimuli can have opposing (modulatory) effects on acute muscle pain. That is, a dual modality of excitatory and inhibitory function resulting in allodynia and analgesia. This result makes CT contributions important for the interaction/processing of nociceptive inputs with LTMR inputs.

However, it should be noted that while the focus remains on low threshold stimuli (tactile and focal cooling) that are known to activate both small and large diameter (A- & C-) low threshold afferent broadly LTMRs, we have not concluded that the allodynia in our animal models is exclusively mediated by C-LTMRs. Currently, the markers used to study these fibres broadly label both myelinated and unmyelinated classes and in the absence of pharmacological tools that reliably distinguish between them. Therefore, the correlation between C-fibres and tactile and cold allodynia following injury appear to be as plausible as those that dominate the literature linking such effect to large diameter fibres. However, the human work clearly shows a proposed link between the modulation of CT and allodynia that is reliant on an affective sensation that survives a compression block of A fibres. Hence, this result and other studies discussing CTs in humans emerging from our lab provide a solid argument of C-fibres as a substantive candidature for allodynia.

In conclusion, primary afferents that normally mediate non-painful sensations may interact with distinct glial cell types to initiate and sustain allodynia. These changes are not only limited to the injured side but also to the non-injured side of the spinal cord. These experiments with dorsal horn glial and peripheral afferent changes allow us to understand the complicated processes that work simultaneously to give rise to low threshold stimulus modality. This data set also raises important questions such as the differences or lack of, in the dorsal horn processing, between different chronic pathological states such as inflammation and injury and whether the specific glial modulatory drugs can have differential effects on the protein expression in the dorsal horn. Moreover, in relation to a bilateral effects, the contralateral side has become a key player in observing minuscule, but important changes related to sensory processing in chronic pain, and hence the traditional use of contralateral side as control may not be suitable for pain studies The dynamic proteins in the dorsal horn responsible for nociceptive behaviours demonstrate that therapeutic approaches should not be limited to classical nociceptors, but also focus on glia and LTMRs, including CTs. Finally, since the CTs have shown a bidirectional processing, the

study supports the idea of a context specific LTMR processing, also possible during an A fibre block, that suggests the involvement of a complex dorsal horn circuitry capable of gaining and gating information as opposed to a hierarchical LTMR network.

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ORIGINAL ARTICLE

Sensory perturbations using suture and sutureless repair of transected median nerve in rats

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ABSTRACT

The effects of changes to cold, mechanical, and heat thresholds following median nerve transection with repair by sutures (Su) or Rose Bengal adhesion (RA) were compared to sham-operated animals. Both nerve-injured groups showed a transient, ipsilateral hyposensitivity to mechanical and heat stimuli followed by a robust and long-lasting hypersensitivity (6–7 weeks) with gradual recovery towards pre-injury levels by 90 days post-repair. Both tactile and thermal hypersensitivity were seen in the contralat-eral limb that was similar in onset but differed in magnitude and resolved more rapidly compared to the injured limb. Prior to injury, no animals showed any signs of aversion to cold plate temperatures of 4–16 C. After injury, animals showed cold allodynia, lasting for 7 weeks in RA-repaired rats before recovering towards pre-injury levels, but were still present at 12 weeks in Su-repaired rats. Additionally, sensory recovery in the RA group was faster compared to the Su group in all behavioural tests. Surprisingly, sham-operated rats showed similar bilateral behavioural changes to all sensory stimuli that were comparable in onset and magnitude to the nerve-injured groups but resolved more quickly com-pared to nerve-injured rats. These results suggest that nerve repair using a sutureless approach produ-ces an accelerated recovery with reduced sensorimotor disturbances compared to direct suturing. They also describe, for the first time, that unilateral forelimb nerve injury produces mirror-image-like sensory perturbations in the contralateral limb, suggesting that the contralateral side is not a true control for sensory testing. The potential mechanisms involved in this altered behaviour are discussed.

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KEYWORD
Bilateral hypersensitivity; Cold allodynia; mechanical allodynia; nerve repair; pain; regeneration

Introduction

Over a million people worldwide suffer peripheral nerve inju-ries each year, most commonly as a result of motor vehicle accidents, violence (stab injuries), work-related injuries (wrist lacerations, sharp objects, carpal tunnel, or repetitive strain injuries) with many requiring surgical repair (Braune and Schady 1993; Galtrey and Fawcett 2007; Huang et al. 2012; Pederson 2014; Barton et al. 2015). The vast majority of these injuries involve the upper limb (Midha 1997; Kouyoumdjian 2006; Eser et al. 2009). Clinically, the most commonly used peripheral nerve repair technique is direct suturing, however, nerve grafting, laser tissue repair, and biocompatible nerve conduits (Lauto et al. 2001) have been developed lately to good effect. A large majority of these repair techniques have been developed in lower limb injury models involving sciatic nerve injury (Lauto et al. 2008; Huang et al. 2012; Geuna 2015) largely because of its ease of access, convenience, and validity of its functional sensorimotor tests (Varej~ao et al. 2004). However, the majority of nerve repairs performed in humans by surgeons involve the upper limb nerves, especially the median and ulnar nerves (Yi et al. 2011). Recovery of hand function is of paramount concern for everyday use in upper limb nerve-injured patients. Likewise, this is also true for rodents where the forelimb has greater dexterity com-pared to the hindlimb and is important for everyday functions like grooming and feeding; forelimb nerve injuries result in profound motor deficits (Bertelli and Mira 1995; Galtrey and Fawcett 2007; Barton et al. 2015). Whilst hindlimb nerve injury models remain the mainstay of preclinical nerve repair research, only a few studies have attempted to establish an upper limb nerve injury model to quantify the sensory–motor changes post-injury (Galtrey and Fawcett 2007; Yi et al. 2011; Cho et al. 2014). These models of median nerve injury can produce signs seen in human nerve injury (Braune and Schady 1993) that appear within days post-injury and lasting many weeks. However, the effects of nerve repair on these sensory perturbations have not been investigated in any detail.

Photochemical tissue bonding (PTB) is a sutureless tech-nique for peripheral nerve repair enabled by incorporating a dye, Rose Bengal, into a thin film of chitosan and activat-ing the dye with a green laser (wavelength ¼ 532 nm), which is selectively absorbed by the dye and causes the tissue to bond to chitosan (Lauto et al. 2012; Barton et al. 2014). Previously, with several histological, imaging, and motor function recovery tests, it has been demonstrated that PTB is an effective method for peripheral nerve repair (Barton et al. 2015). However, the effects on sensory altera-tions following a median nerve repair using the novel method of PTB were not described. To explore the advan-tages of this novel method of nerve repair with PTB over traditional suturing methods, we quantified the surgery-induced perturbations of sensory function to cold, tactile,

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and heat sensitivity over a period of 3 months in rats sub-ject to two different nerve repair methods and compared them to sensorimotor changes in sham-operated animals. We hypothesized that the PTB repair method is a better technique than the traditional nerve suturing method in terms of functional sensory recovery in experimental rats.

Experimental procedures

Surgery

All animal experiments were approved by the University of Western Sydney animal care and ethics committee (ACEC: A8900) and were conducted in accordance with the ethical standards of the university. Healthy, adult female Long Evans rats (n ¼ 30; 180–220 g) were used for the surgery and the behavioural testing for the 90-day period and were housed in the University of Western Sydney animal house (School of Medicine, Campbelltown, NSW, Australia) during the entire study.

Surgical anaesthesia was induced with 4% isofluorane (Sigma-Aldrich, Sydney, Australia) and maintained by 2.5% isofluorane in 100% oxygen. Under sterile conditions, using an Olympus operating microscope, the left median nerve was exposed through a ventromedial skin incision extending from the pectoralis major muscle to the cubital fossa. The underly-ing muscles of the axillary region were separated by blunt dissection to reveal the median nerve which was separated from the surrounding connective tissue at the brachial plexus at the level of the pectoralis major to the cubital region. Each animal was allocated randomly into one of three experi-mental groups (N ¼ 10 per group, Table 1). In group 1, follow-ing complete nerve transection, a strip (5 3 mm^2) of Rose Bengal chitosan adhesive (RA) was wrapped around the approximated nerve to form a cuff and laser irradiated at 532 nm with a fluence of 133 J/cm^2, as per the PTB meth-ods described previously (Lauto et al. 2012; Barton et al. 2015). In group 2, following complete nerve transection, nerve ends were approximated using 10-0 monofilament nylon Su (Ethicon) as described previously (Lauto et al. 1997; Barton et al. 2015). Finally, in group 3, the PTB procedure (including laser irradiation) was performed as in group 1 but without transecting the nerve. In all groups, the muscles were realigned and the skin closed with surgical staples and the paws treated with self-mutilation deterrent antiseptic solution (VirBac Animal Health, Milperra, Australia). All animals were allowed to recover for 1 week in the animal facility before commencing behavioural experiments.

Table 1. Procedures performed on each group during surgery.

Group	Procedure	Nerve cut	Laser irradiation
1	Photo tissue bonding with Rose Bengal adhesive	Yes	Yes
2	Nerve suture	Yes	No
3	Sham	No	Yes

N ¼ 10/group.

Behavioural tests

Behavioural tests were performed twice a week for at least 1 week prior to surgery (baseline conditioning) and twice a week following surgery by one observer blinded to the treat-ment groups. Animals were habituated to their surroundings for 5–10 min before testing commenced. On any given day, the order of tests was randomized and a 5–10 min rest inter-val was given between successive tests.

Cold and warm plate tests

The rats were placed in a clear Plexiglas chamber where the temperature of the metal base was 25 C. For cold plate test-ing, after 3 min, the temperature was rapidly lowered to 16 C and the animal's behaviour was observed for 3 min for signs of pain-like behaviour such as escape rearing, avoiding contact with the cold plate, suspension of the affected forelimb, licking of the paw, lack of grooming and exploration vocalization, or freezing behaviour. Three or more of the signs needed to be elicited for a temperature to be considered painful. After 3 min the plate temperature was returned to 25 C for 30 s before moving to a lower temperature. In the absence of a distinct response, the temperature was lowered further by 2 C (range 16–4 C). Once pain-like behaviour was observed, the tempera-ture was increased to a higher temperature until the behaviour disappeared (maximum 16 C) in order to confirm the thresh-old measurement (methods of limits).

For warm plate testing the temperatures were raised from 20 to 36 C in 2 C increments and behaviour assessed as described above.

Withdrawal threshold tests

All animals were allowed to acclimatize (5 min) to the new chamber before commencing each test. A mechanical stimu-lus was delivered to the plantar surface of the glabrous median nerve territory of the left or right forepaw using a dynamic plantar aesthesiometer device (Bioseb, Chaville, France) to measure tactile thresholds. The force increased at 10 g/s in 0.5 g steps until the animal withdrew its paw or a maximal force of 50 g was achieved. An infrared plantar anal-gesia instrument (Hargreaves apparatus, Bioseb, power flux: 0.64 mW/cm^2, 15 s cut-off time) was used to measure thermal withdrawal reflexes (Hargreaves et al. 1988; Montagne-clavel and Oliveras 1996). On any test day, three consecutive meas-urements for each forepaw were recorded, separated by 30-s rest intervals, and averaged for both types of stimuli.

Statistical analysis

All data is expressed as mean 6 standard error of the mean (SEM). All statistical tests were carried out with GraphPad Prism version 6 software (La Jolla, CA, USA). Two-way repeated meas-ures ANOVA was used to analyse data for the time course with all groups and a Dunnett post hoc test for multiple compari-sons with the criterion for significance set at $p < 0.05$.

In Figures 2 and 3, the dashed horizontal lines were included to indicate the boundaries above and below where

the mean values differed significantly from the control values. The duration of sustained hyperalgesia, prior to the onset of a progressive recovery was determined by the point at which three or more successive time points differed significantly from the maximum hyperalgesia reported.

Results

Immediately following surgery all animals were returned to communal housing and, consistent with earlier observations (Barton et al. 2015), all animals were in good health and displayed no signs of infection or autotomy behaviour.

Responses to cooling

Prior to surgery rats displayed no preference for a plate held at room temperature or a cold plate (16–4 C), nor did they display any behavioural changes even when the temperature of the plate was reduced to 4 C. In contrast, 7 days following surgery marked behavioural responses were observed when the plate temperature was reduced to <16 C (Figure 1) across all three groups, indicating that previous indifference to cooling had been replaced by a marked hypersensitivity to cooling and the emergence of cold allodynia. At the onset of hypersensitivity (day 7), the threshold temperature required to evoke a response, displayed a rank order, in which Su (16.0 6 0.0 C) >rose adhesive (RA, 14.0 6 0.0 C) >sham (12.0 6 0.0 C). This initial hypersensitivity was preserved from days 7 to 19 following surgery, and was followed by a progressive recovery over 6 weeks where successively lower temperatures were required to evoke cold allodynia (16–10 C) in all three groups ($F = 1995$; $p < 0.0001$). From 42 days post-surgery, all groups showed an allodynic response to progressively lower temperatures indicating a progression towards cold hyperalgesia (i.e., <10 C, $F = 3218$; $p < 0.0001$), especially in sham and RA nerve-injured animals. By day 79, Su-treated nerve-injured animals had a cold threshold that was now considered hyperalgesic (i.e., below <10 C), whereas sham-operated and RA-treated nerve-injured animals showed a lack of allodynia responses at 4 C suggest-ive of return towards pre-injury behavioural responses. Lower temperatures were not tested. The Su group were still signifi-cantly cold sensitive at 3 months post-injury compared to other groups suggesting that RB treatment improves thermal recovery.

Responses to mechanical stimuli

As shown in Figure 2, prior to surgery, the baseline mechanical withdrawal thresholds did not significantly differ between successive test days, between left and right forepaws or between experimental groups (27.3 6 1.7 g, range 26.0–28.6 g, $p > 0.05$).

Following surgery, marked differences in the onset and duration of mechanical sensitivity were observed across all experimental groups. In the sham group (black lines), where the surgical exposure of the left median nerve was identical to all other groups, but the nerve remained intact, an immediate (day 7) bilateral reduction in the withdrawal thresholds was observed (left: 6.6 61.8 g, right: 8.4 6 2.2 g, $p < 0.001$), suggestive of hypersensitivity, that remained for up to 40 days before progressively returning to pre-surgery levels on the contralateral side and ipsilateral side by day 55. Although the peak intensity of the cutaneous allodynia did not differ between the nerve-injured (RA and Su) groups to the sham group ($p < 0.001$) on the ipsilateral side, the duration of the

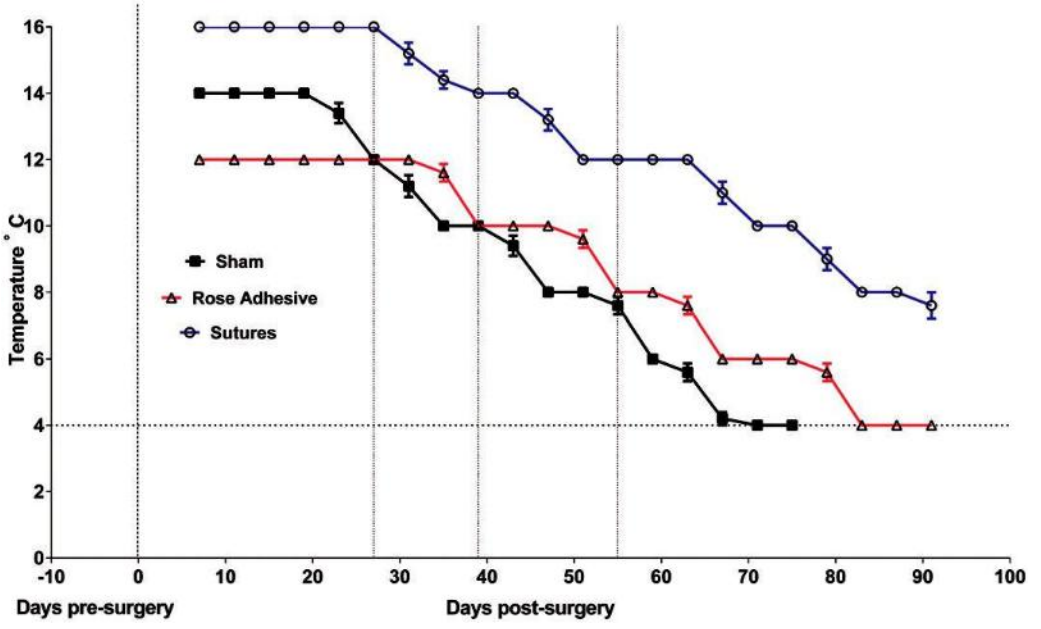

Figure 1. Graph of minimal plate temperature (C) required to elicit cold hypersensitivity. Prior to surgery, all animals were unaffected by plate temperatures between 4 and 16 C. In contrast, sham (black line, filled symbol), RA (red line, open symbol), and Su groups (blue line, open symbol) displayed a marked sensitivity to previ-ously innocuous reductions in temperatures, suggestive of cold allodynia. N ¼ 10/group; mean 6SEM. Vertical lines represent the cut-off temperature determined by humane endpoints. First vertical line from the left represents day 0 or surgery day and subsequent vertical lines represent time points at which sham and RA groups overlapped.

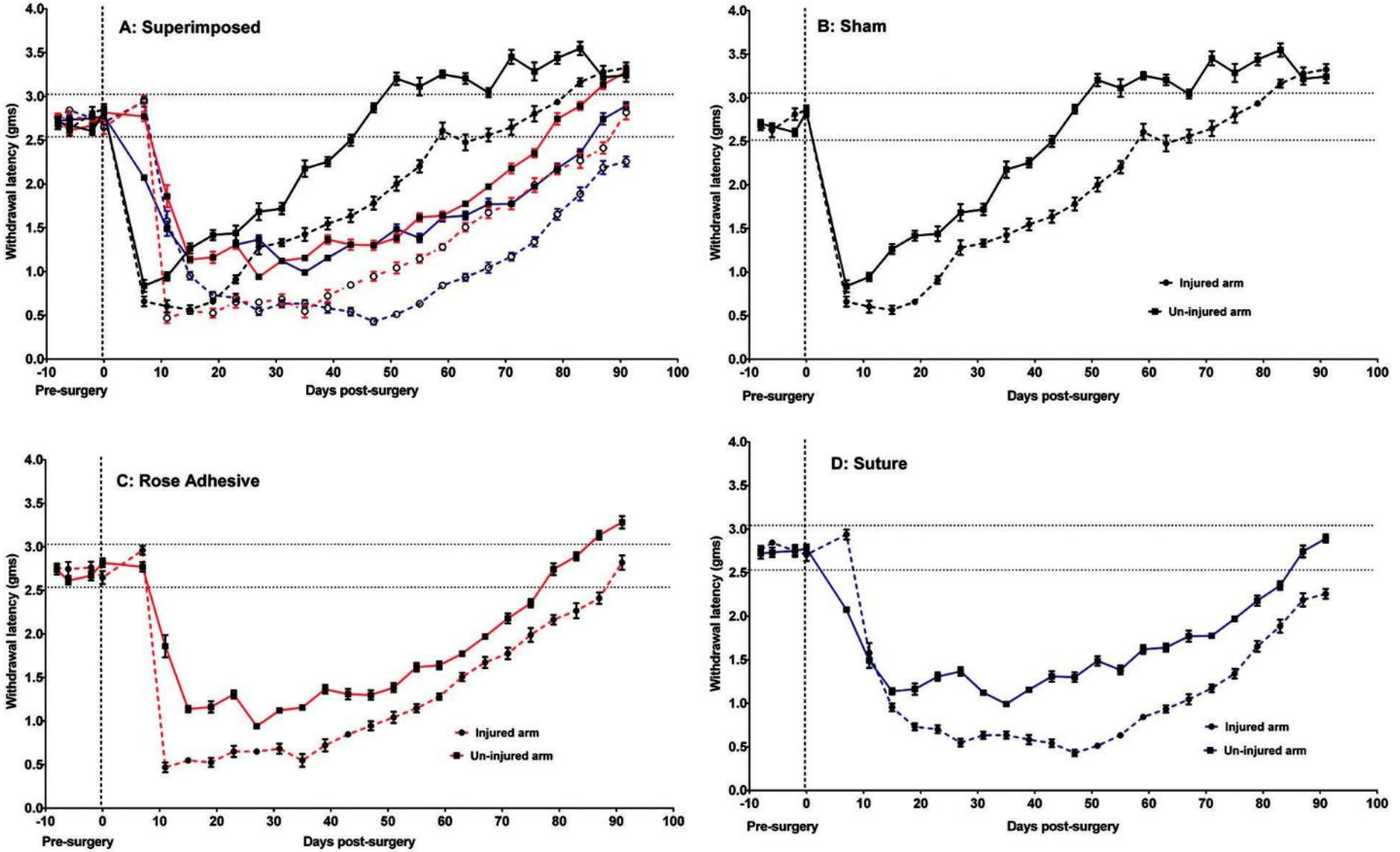

Figure 2. Withdrawal latencies to tactile stimuli. Mean grams of force to induce a flexion withdrawal to a punctate stimulus are shown in sham-operated (B, black), RA (C, red), and Su (D, blue) groups on the operated (left, dashed lines) and intact (right, solid lines) forelimbs prior to and following surgery. From top left: superimposed data (A) for all groups (top to bottom: sham right, sham left, RA right, Su right, RA left & Su left); separated data for sham (B), RA (C), and Su (D) groups are shown (n ¼ 10/group, mean 6 SEM). In each figure the dashed horizontal lines indicate the boundaries above and below which the mean values differed significantly from the control values.

sustained allodynia (days 7–21) in the operated forelimb was prolonged as the onset of the recovery phase (and subse-quent hypoalgesia) was delayed by 10–15 days as com-pared to the contralateral limb. In contrast, in groups 2 and 3, where the left median nerve was transected and repaired, the initial (day 7) response of the ipsilateral limb was character-ized by a hyposensitivity to tactile stimulation (RA: 29.6 6 1.6 g and Su: 29.4 6 1.8 g; $p < 0.001$) compared to pre-injury. In the RA group, the contralateral limb withdrawal reflex was unaltered at 7 days but in the Su group contralat-eral hypersensitivity was apparent at this time point. This transient hyposensitivity was followed by the emergence of a bilateral reduction in withdrawal thresholds (RA: left:
4.7 6 1.7 g; right: 18.6 64.0 g and Su: left: 15.8 6 3.5 g; right:
14.9 6 2.7 g; $p < 0.001$) at day 11 that plateaued by day 14 (at different time points, respectively, for the ipsilateral and contralateral limbs) and persisted throughout days 14–42 before the onset of a bilateral gradual recovery phase (days 40–90). In both RA and Su groups the recovery of hypersensi-tivity lagged by 10 days in the repaired forelimb compared to the contralateral limb. Furthermore, in those animals in which the nerve was repaired using Su, the onset of recovery was later compared to those repaired using RA by around 10 days. Intriguingly, once the recovery phase had commenced the rate of recovery (change in threshold values per day) did not differ between experimental groups.

Responses to heat stimuli

The use of a rapidly rising, intense, and ultimately noxious heat stimulus produced a similar tri-phasic response across all three groups, characterized by a variable, transient early response, a sustained bilateral hypersensitivity, and finally a normalization of response (Figure 3). Prior to surgery, with-drawal latencies did not significantly differ between succes-sive tests, between forepaws or experimental groups (Figure 3, 6.4 6 0.2 s, range 5.9–6.7 s, $p > 0.5$). Post-injury, in the sham-operated group 7 days after surgery, a significant (1.7 60.2 s; $p < 0.001$) and sustained (up to 63 days) reduction in withdrawal latency was observed in the injured forelimb that preceded a delayed (4 week) and less marked (5.3 6 0.2 s; $p < 0.01$) reduction in withdrawal thresholds in the uninjured contralateral forelimb.

Post-injury, both nerve lesion groups displayed a transient hyposensitivity for 7–11 days following nerve repair with RA or Su in the injured and repaired, left limb (RA: 8.3 6 0.3 s, Su: 8.5 6 0.3 s, $p < 0.001$) that preceded a rapid (days 11–15) reduction in withdrawal latencies (RA: 1.8 6 0.2 s, Su: 1.9 6 0.1 s, $p < 0.001$) which was maintained over the next 6 weeks. This hypersensitivity was observed bilaterally but was less marked (RA: 4.0 60.2 s, Su: 4.1 6 0.2 s) and slower in onset in the uninjured limb (days 14–19) than in the injured limb. The recovery timelines did not differ significantly on the injured limbs (days 47–90) and the un-injured limbs (days 55–90) between both RA and Su groups (Figure 3).

Rats displayed no preference for a plate held at room tem-perature or showed any aversive responses when the warm plate temperature was varied between 20 and 36 C. Moreover, this behaviour did not change after nerve injury and repair indicating an absence of warm allodynia.

Discussion

Nerve injury activates molecular cascades that prime the nerve to regenerate (reviewed by Chan et al. 2014). Complete lesions require physical repair, and there are a variety of sur-gical methods available to facilitate functional recovery (reviewed in Barton et al. 2014). Previously, it was demon-strated that sutureless repair of a complete nerve transection accelerated motor functional recovery (Barton et al. 2015) as compared to sutured nerves. The results of the present behavioural study compliment and extend those previous observations by showing accelerated sensory recovery that is also more rapid in RA-repaired rats compared to Su-repaired rats. Median nerve injury produced a tri-phasic response consisting of a transient hyposensitivity, followed by profound hypersensitivity to both thermal and mechanical stimuli that lasted around 6 weeks, before resolving over the following 6 weeks. Interestingly, similar observations were seen in sham-operated rats. Moreover, in all groups such effects were bilateral. As far as we are aware, this is the first report of contralateral behavioural changes in models of nerve injury, and suggests that the contralateral limb is not a true control of sensory thresholds.

The sciatic nerve of the hindlimb is the most common injury model used to explore novel surgical and/or biomate-rial strategies to improve nerve regeneration (Huang et al. 2012; Geuna 2015). More recently, the median nerve has become a popular model for nerve repair (Sinis et al. 2011; Barton et al. 2014). This is because in rodents, the median nerve innervates all the finger flexors and there are several well-described motor behavioural tasks that clearly define the progress of motor recovery (Bertelli and Mira 1995; Galtrey and Fawcett 2007; Jager et al. 2014). Much less attention has been focused on sensory deficits and pain-like behaviour, in part because previous studies have used complete nerve sec-tion (median and ulnar) and this produces complete hyposen-sitivity (Bertelli and Mira 1995; Galtrey and Fawcett 2007). Two previous studies have focused on sensory disturbances following nerve damage. Yi et al. (2011) developed a model of partial median and ulnar nerve injury which resulted in unilateral mechanical, thermal, and cold hypersensitivity with a time course very similar to the results of this study. Interestingly, the most sensitive and prolonged behavioural change was associated with cold allodynia but no contralat-eral changes were observed. Cho et al. (2014) used a median nerve model using nerve ligation at the forelimb (beneath the elbow) and observed changes over a 1-month period. Like Yi et al. (2011) and in our study, they also observed hypersensitivity to heat, cold, and mechanical stimuli. Again, no contralateral changes were seen in any stimulus except for a transient cold allodynia 4 days after injury. One clear differ-ence between this study and those of Yi et al. (2011) and Cho et al. (2014) is the ipsilateral hyposensitivity that occurred during the first week after injury, a time point that was not tested in this study. This can be explained by differences in

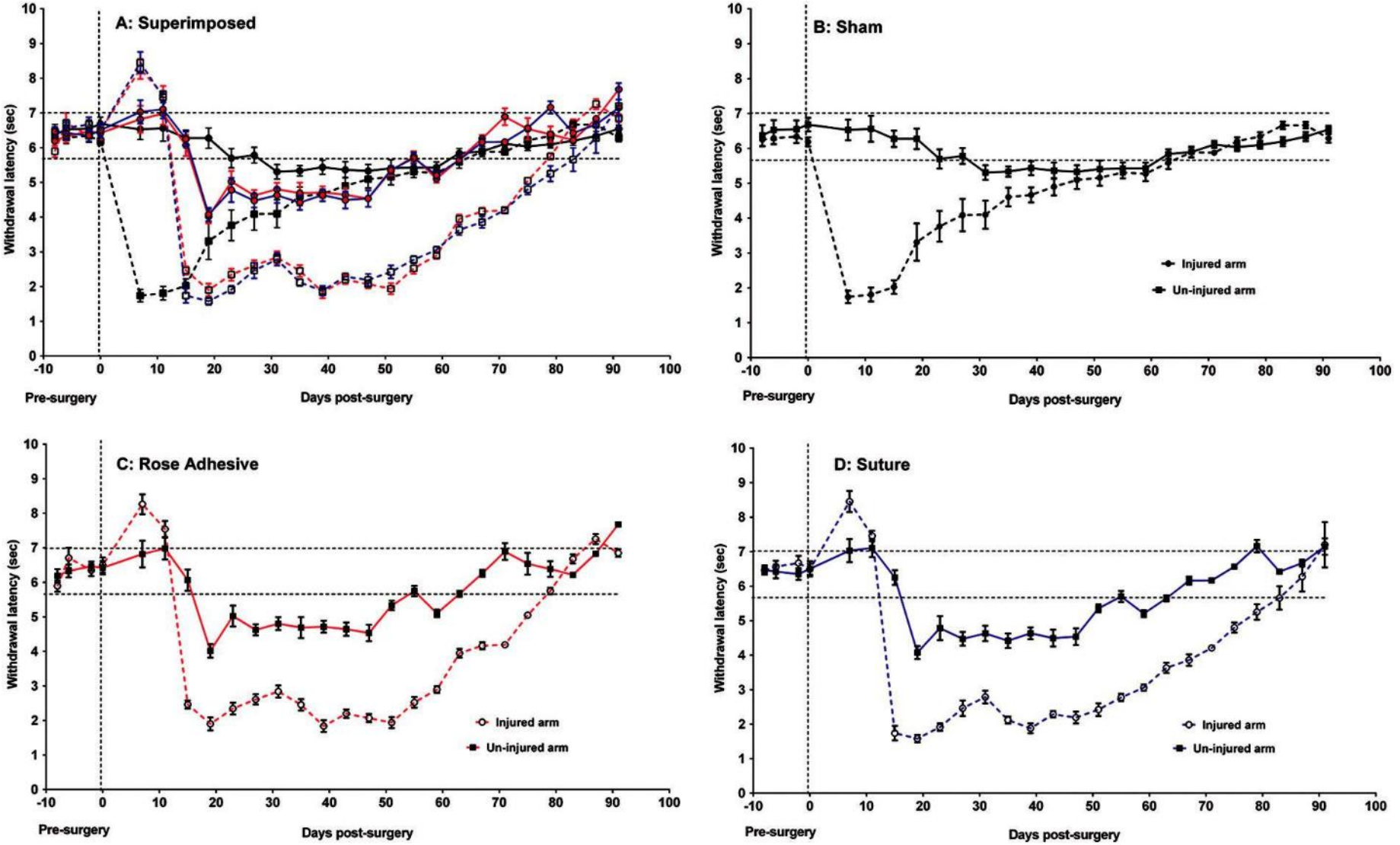

Figure 3. Withdrawal latencies to thermal stimuli. Mean times to induce a flexion withdrawal to a thermal stimulus are shown for sham-operated (black), RA (red), and Su (blue) groups on the operated (left, dashed lines) and intact (right, solid lines) forelimbs prior to and following surgery. From top left: superimposed data (A) for all groups, sham (B), RA (C), and Su (D) groups are shown (n ¼ 10/group, mean 6SEM). In each figure the dashed hori-zontal lines indicate the boundaries above and below which the mean values differed significantly from the control values.

nerve model in the case of Yi et al. (2011) where the glabrous forepaw still has some peripheral innervation but in the case of Cho et al. (2014) and our model, the effects could be due to a contribution of adjacent nerves, that is, radial and ulnar, that remained intact in both models.

That the hyposensitivity of nerve transection and repair is replaced by hypersensitivity after a week is consistent with a peripheral sprouting mechanism being involved in evoked pain-like behaviours. Nerve injury induces Wallerian degener-ation which causes the production of trophic factors like nerve growth factor (NGF) in the peripheral skin territory (Mearow et al. 1993) that then leads to peripheral sprouting of C fibres into the denervated territories (Inbal et al. 1987; Wiesenfeld-Hallin et al. 1989; Brennan et al. 1996). Activation of these fibres can result in hypersensitivity. Additionally, the nerve injury generates a central microglial response which can spread to the adjacent intact nerve territory in the central nervous system (CNS) (Beggs and Salter 2007). Release of pro-inflammatory mediators from glia can induce central sensitiza-tion to initiate and maintain the hypersensitivity (Milligan and Watkins 2009; Beggs and Salter 2013; Tsuda et al. 2013). As the nerve regenerates, peripheral sprouts retract (Devor et al. 1979; Diamond et al. 1992) and as peripheral reinnervation occurs the hypersensitivity resolves.

One clear and striking observation in this study is the bilat-eral hypersensitivity that occurs after unilateral injury. This is reminiscent of mirror-image pain reported in other animal (Koltzenburg et al. 1999; Watkins et al. 2001; Huang and Yu 2010; Jancalek 2011) and human (Konopka et al. 2012) studies following unilateral nerve injury. The mechanisms that con-tribute to this type of pain have been speculated to fall into three general categories: humoral, peripheral, or central mechanisms, with most evidence favouring central mecha-nisms. Research suggested that spinal cord glial cells were intimately involved in mirror-image pain as a result of trans-neuroglial communication. However, an elegant study by Cheng et al. (2014) demonstrated that nerve injury induced a rapid, ipsilateral upregulation of tumour necrosis factor alpha (TNFa) which then diffused in the cerebrospinal fluid (CSF) to stimulate contralateral dorsal root ganglia (DRG) satellite cells to produce NGF that triggered C fibre hyperexcitability lead-ing to mechanical hypersensitivity. This study also suggested that DRG satellite cells contributed to onset of the pain while the spinal astrocytes were responsible for its maintenance. A very recent study has added a further layer of complexity to the mechanisms involved by demonstrating that different immune cell types contribute to mechanical pain in male and female mice (Sorge et al. 2015); microglia are primarily involved in males but not females where T cells are important.

The bilateral hypersensitivity was also observed in sham-operated animals. The onset of the behavioural changes was faster in the ipsilateral limb compared to the nerve-injured groups, but the magnitude of the behaviour was similar. However, resolution was faster compared to the other groups. The behaviour elicited by sham surgery can be considered to be similar to acute post-surgical pain experienced by humans and animals (Perkins and Kehlet 2000) as a result of periph-eral and central sensitization of afferents and neurons (Fitzgerald 1979; Kawamata et al. 2005). Post-operative pain usually only lasts a few days but the behaviour lasted several weeks. This indicates that hypersensitivity persists even when the cutaneous wound appears to have healed. The sham-operated animals had RA applied to an intact nerve and it is possible that the RA elicited a central immune response to produce the observed behaviour. Whilst the mechanisms regarding this behaviour remain unclear, they do suggest that sham-operated animals are not true control groups and so changes in comparison to sham groups may be underestimated.

In this study, the most obvious and robust indicator of dis-comfort and hypersensitivity in all operated rats was observed when the temperature fell below 16 C, a range that normally failed to evoke any aversive responses prior to surgery. Moreover, cold allodynia never fully reversed in any of the groups during the 90-day testing period. These results are similar to the cold intolerance experienced by patients with nerve trauma (Novak et al. 2012). The assessment of cold behaviour used in this study differs from more traditional tests such as acetone cooling, ice baths, or cold plates (Allchorne et al. 2005). The use of a dynamic cooling para-digm from a thermally neutral temperature to cool or cold temperatures allowed us to monitor not one, but several aspects of animal behaviour, and is akin to psychophysical data used in human experiments (Samour et al. 2015) to measure cold allodynia. The clear change in behaviour was easily recognizable from other types of exploratory behaviour and reflex withdrawal methods that have been traditionally used. Additionally, more variable behaviour was found with increasing temperatures. When a similar warming paradigm was used, animals remained indifferent to temperature increases from 20 to 36 C indicating lack of warm allodynia. In the Hargreaves test, which measures the noxious threshold for flexion withdrawal to heat, thermal hypersensitivity was detected.

Allodynia has been traditionally ascribed to myelinated afferents and sensitized nociceptors (Campbell et al. 1988), and innocuous cold sensibility is often attributed to Ad fibres (Samour et al. 2015). However, recent work has shown that C low-threshold mechanoreceptors (CLTMRs) can also contribute to allodynia (Nagi et al. 2011; Nagi and Mahns 2013a, 2013b). C mechanoreceptors have low mechanical thresholds, responding to light touch to the skin (Zotterman 1939; Maruhashi et al. 1952; Douglas and Ritchie 1957) as well as evaporative cooling (Nordin 1990). Extending this dual action, recent human (Nagi et al. 2015; Samour et al. 2015) and ani-mal studies (Franc¸ois et al. 2015) have shown that not only the effect evoked by mechanical stimuli was diminished but also cooling-evoked allodynia was prevented by selective blocking of CLTMRs suggesting that this class of afferent may mediate tactile, as well as cold, hypersensitivity in these groups.

In summary, this behavioural study has shown that median nerve injury produces rapid and long-lasting, bilateral hyper-sensitivity to thermal and mechanical stimuli. Surgical repair by RA produces a more rapid recovery of sensory function compared to Su-repaired nerves. This suggests that a

sutureless approach to nerve repair may have more beneficial effects compared to traditional surgical methods.

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Disclosure statement

The authors disclose that there are no conflicts of interest.

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Paper II

Implications of functional proteomics on the contralateral dorsal horn in the context of bilateral allodynia and glial changes post-injury

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Abstract

Unilateral median nerve injury produces a long lasting bilateral tactile and cold allodynia - where previously non-painful mechanical stimulus and cooling temperature can become painful. Glial role in the modulation of painful responses have been long established. We used minocycline, a microglial inhibitor, to modulate the observed bilateral allodynia and study the changes at the cellular and proteomic level in the ipsi- and contra-lateral dorsal horn. An enhanced astrocytic expression in the injured and minocycline treated group, in particular at the contralateral side suggested a greater role of astrocytes in generalised allodynia. The proteomic profiling of the dorsal horn in these groups showed that the astrocytic marker GFAP (highest MOWSE score) followed the same pattern as the immune-labelling. Therefore, modulating the microglial activity and its subsequent impact on bilateral pain processing, the importance of astrocytic function in central sensitisation is highlighted. Protein changes responsible for the maintenance of a heightened energy, increased glucose consumption and glutamate re-uptake corresponding to the behavioural hypersensitivity are also observed. The 2D gel analysis in combination with mass spectroscopy of the injured and contralateral dorsal horn revealed several important proteins that are up-and down-regulated in various functional categories. The top modulated proteins in the process of ameliorating allodynia may serve as future pain targets. Due to the nature of central involvement, this study also argues against the use of contralateral side as a control for studying pain physiology.

Significance Statement

- Minocycline administration in nerve injured rodents ameliorates injury induced bilateral allodynia
- The bilateral allodynia corresponds to the glial changes in the dorsal horn lamina II, known to process allodynia.
- This glial-behavioural modulation using minocycline is quantified at the molecular-proteomic level within the dorsal horn using 2D gel electrophoresis and mass spectrometry.
- Marked astrocyte expression on the contralateral side and the GFAP proteomic expression establishes the link between central sensitisation and astrocytic function.
- Several metabolic proteins expressed bilaterally reveals the role of glia in the processing of pain involving ATP, glucose breakdown and glutamate buffering.
- The contralateral horn profiling yielded important low volume proteins that may be functionally responsible for bilateral allodynia and are otherwise masqueraded on the injured side.

Introduction

Nerve injuries produce chronic long-lasting pain that often exists after the initial site of injury is healed. Symptoms such as allodynia, where a non-painful stimulus is perceived as painful is one of the most common injury induced hypersensitivities. The change in sensory function that underpins allodynia is not reliant on the classical pain afferents such as C nociceptors or high threshold fibres, but can include inputs from low threshold mechano-receptors (LTMRs) that normally mediate non-painful touch sensations, e.g. Aβ fibres (A-LTMRs) [12; 35], and the unmyelinated C-LTMRs [21; 36; 42; 45]. The participation of LTMRs in this aspect of pain processing may, in part, explain the limited efficacy of many treatments that have a large number of side effects [5; 26; 66; 67].

In humans and animals, these sensory perturbations are often generalised and extend bilaterally or to other uninjured areas of the body [9; 15; 16; 51]. Once the peripheral site of injury is healed, the maintenance of pain is no longer reliant on the injured site, but is attributed to central process (e.g. spinal dorsal horn). The mechanism that causes the spreading of pain to the contralateral side to the injury has been attributed hitherto to glial activation and pro-inflammatory release [17; 51; 79; 82]. However, the precise nature of this allodynia by way of mirrored topography suggests the neural involvement. Previously, some anatomical studies have confirmed this symmetry of the spinal nerve connectomes [27-29], while another study has confirmed long lasting structural changes on the contralateral dorsal horn due to a unilateral nerve lesion [20].

In the last decade, increasing evidence from animals and humans suggests that allodynia is not solely reliant on myelinated low-threshold A mechano-receptors (A-LTMRs) but low threshold C tactile fibres (C-LTMRs), known for affective touch, are also involved [17; 34; 37; 39; 55]. In the dorsal horn, where these LTMRs terminate, conjointly with other surrounding cells, i.e. interneurons and post-synaptic connections, non-painful and painful touch (allodynia) is regulated via a proteomic signalling system like the GABA-ergic inhibitory release [1; 23; 47]. In addition, the glial immune cells such as the microglia and the astrocytes of the dorsal horn play a vital role in the regulation and maintenance of these sensations that include the process of ATP breakdown, glutamate re-uptake and glucose metabolism [3; 19; 43; 48; 51; 54; 63; 64].

Therefore, at the spinal level, bilateral allodynia is mediated by a combination of afferent processing related to origin of afferent input, inter-neuronal relay, glial cell function and signalling by proteins and molecules. Studying the changes in this concoction on the injured and the contra-lateral dorsal horn of the corresponding spinal segment can help understand how changes in structure and protein expression leads to changes for pain processing. Moreover, using a glial modulatory drug, such as minocycline, which produced a suppression of injury induced bilateral glial cell activation and behavioural hypersensitivity

(tactile and cold allodynia), was used to identify a host of targets that can be correlated with perturbed function and identify key proteomic changes that are associated with the emergence of post-injury hypersensitivities.

The aim of this study was to determine whether the concomitant administration of minocycline supresses the nerve injury induced bilateral tactile and cold allodynia, and characterise the pattern of associated changes in glial cell function along with the pattern of protein expression in the dorsal horn. Minocycline, a weak anti-inflammatory, otherwise used to treat acne has gained increased attention due to its anti-allodynic properties [38; 44; 49; 50]. Spinal proteomic profiling of injured and contra-lateral dorsal horn with 2D gel electrophoresis and mass spectrometry has allowed to trace the nerve injury induced changes and identify those that are modulated by minocycline.

Materials & Methods

Experiments were conducted on adult male and female Long Evans rodents weighing 120–150 g. The experiments were approved by the Animal Ethics Committee of the university (Approval number: A10622). IASP (International association for the study of pain) guidelines for pain research in conscious animals were followed [71] and all efforts were made to minimize the suffering and the number of animals used for the experiments.

Experimental design

All animals (n=24, 12 male + 12 female) underwent sensory behavioural testing prior to and following allocation to the control and nerve crush experimental groups. As shown in table 1, half the animals from each group were treated with minocycline and half with a vehicle (0.9% saline). All behavioural, histological and proteomic assessments were conducted blind to the experimental group and treatment.

Table 1: Distribution of animals in each experimental group

Intervention / treatment	Control	Nerve crush
Vehicle (0.9% saline)	6	6
Minocycline (40 mg/kg)	6	6

Following completion of behavioural testing on day 7, three animals from each group were allocated to immunohistochemistry and three to proteomic analysis. The proteomic and histological data shown is only for naïve saline treated, injured saline treated and injured minocycline treated animals as the focus was on the modulation of allodynia post injury due to minocycline, for which, the saline treated naïve animals was an appropriate control.

Unilateral median nerve crush

Following initial sensory testing, rodents were anesthetised (2-4% Isofluorane in 100% oxygen) and under sterile conditions, the left median nerve was exposed using methods described previously [6; 57] and the nerve crushed using sterile forceps midway between its origin and the cubital fossa for 10 seconds so that the crushed nerve was translucent but not detached. After the injury, the wound was closed with sterile wound clips and washed with iodine solution.

Sensory tests

Behavioural changes were measured using sensory testing protocols for aversive-ness to heat, mechanical (gradient: non-painful to painful) and previously non-painful cold stimulus as described previously [57]. For all animals, behavioural testing was performed twice, prior to (conditioning) and on alternate days, following surgery (day 0). Based on pilot

behavioural experiments, animal were sacrificed on day 8 where peak sensory hypersensitivity had been observed.

Minocycline treatment

Minocycline hydrochloride (Sigma-Aldrich, Gilliangham, Dorset, UK) was dissolved in 0.9% saline (vehicle) and injected into the intra-peritoneum (i.p.) at a dose of 40 mg/kg [14]. Injections were given immediately by following nerve injury (day 0) and every day for 7 days post-surgery. Vehicle-treated rodents were injected with the same volume of 0.9% saline using the same regimen. All behavioural assessments were made at least 24 hours after i.p. injection.

Immuno-labelling

Rodents were overdosed with sodium pentobarbital and trans-cardially perfused with phosphate buffered saline (PBS, 0.1M, pH 7.4) followed by 4% paraformaldehyde in 0.1M PBS (PFA). The spinal cord C6-C7 segments were carefully excised (corresponding to the central termination of the median nerve). A 32-gauge needle was used to mark the contralateral ventral horn. Tissue was post-fixed in PFA for 3h, then soaked in 10% sucrose in PBS for 1h at room temperature and stored in 30% sucrose in PBS at 4°C until sliced. For slicing, tissues were embedded in an optimal cutting temperature compound (OCT) and cut into coronal sections within a freezing microtome (CM1900, Leica, Nussloch, Germany) at 60µm. Free floating sections were washed with PBS before treatment with antibodies. Following antibody treatment, all sections were rinsed at least thrice and following mounting (HistoBond, Germany), sections were treated with Vectashield hardset anti-fade mounting medium with DAPI (Vector Laboratories, United States) to visualise cell nuclei.

Glial immuno-staining

Triplicate C7 sections from the cord of each animal were treated with anti-Iba1 (raised in rabbit, Cat# 019-19741, Novachem, Australia) and anti-GFAP (raised in mouse, Cat# MAB360, Millipore, Australia) antibodies at 1:1000 dilution (in 0.1M PBS + 0.2% Triton at 4°C) for 48 hours and visualised with Alexa Fluor® 488 (goat anti rabbit) and Alexa Fluor® 594 (goat anti mouse, Abcam, Australia) respectively, following a 2 hour incubation.

Superficial lamina immuno-staining

Anti-CGRP, a peptidergic marker of laminae I-II, was used to determine the boundaries of lamina II inner (LII_i). Every 5^{th} section from the C7 segment was treated with anti-CGRP (raised in mouse, Cat# PC205L, Millipore, Australia) at 1:1000 dilution (in 0.1M PBS + 0.2% Triton at 4°C for 48 hours) and visualised with Alexa Fluor® 488 (goat anti-mouse, Abcam, Australia) following a 2 hour incubation to determine the area of the superficial laminae in the cord for stereological analysis.

Microscopy

Using published morphological and cyto-architectonic landmarks of the cervical dorsal horn [56] and the observed anti-CGRP staining of the C7 spinal cord, the laminar boundaries of

the spinal cord grey matter were defined at 10x. Lamina II was divided into lamina II inner (LII_i) and outer (LII_o). Images were taken for all antibodies' staining at 5x and 10x using digital camera (Hamamatsu) connected to a Leica fluorescent microscope for analysis. Ipsilateral and contralateral sides were identified using the needle-mark (hole) in contralateral ventral horn.

For each animal, in LII_i, labelled $Iba1^+$ cells (at 40x) were counted using Stereo Investigator (MBF Bioscience, United States) and $GFAP^+$ labelling was quantified using a measure of its fluorescence intensity using ImageJ software (NIH, United States). In each animal, data from lamina II_i in triplicate sections were averaged and the pooled results from three animals were plotted as mean $\pm$ standard error of the mean (SEM).

2D Gel Electrophoresis

Chemicals

All consumables were of electrophoresis grade or higher quality. Electrophoresis consumables including acrylamide, tris-glycine SDS buffer, were purchased from Ameresco Inc. (Solon, OH). Double distilled water (ddH_2O) was used for all reagents prepared in this study.

Dorsal horn tissue extraction

On day 8, under deep surgical anaesthesia (2-4% Isofluorane in 100% oxygen), animals were perfused with ice cold 0.9% saline, followed by ice cold PBS. The C6-C7 segments of the spinal cord were harvested and cut into left and right; dorsal and ventral quadrants yielding four sections of tissue from each animal. The left and right dorsal horn quadrants were snap frozen in liquid nitrogen and stored at -80°C. All types of tissue handling was conducted on ice.

Separation of membrane and soluble fractions of DH

While left (injured) and right (contra-lateral) dorsal horn were analysed separately, the samples from 3 animals (e.g. left dorsal horn) were pooled within each experimental group due to limited tissue size in each quadrant. These samples were pulverised in a chilled self-sealing Teflon chamber (2mL internal volume) containing a 9 mm steel ball bearing using a Mikro-dismembrator S model (Sartorius Stedim Biotech GmbH, Gottingen, Germany) for 30-45s at 2000 rpm.

While on ice, each powdered sample was suspended and vortexed in three volumes of chilled hypotonic lysis buffer [20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 with 1 × PIC (PIC; 2µg/mL aprotinin, pepstatin and leupeptin)] with the same volume of chilled 2 × PBS and 2 × PIC. Separation into membrane and soluble compartments was performed using the urea buffer exchange methods used earlier [24].

Protein concentration assay

The concentrations of total membrane and soluble samples were measured against a Bovine serum albumin (BSA) standard using the EZ-Q protein quantitation assay (Molecular Probes, Eugene, Oregon) and imaged using LAS-4000 imager and analysed using Multi Gauge software v3.0 (FUJIFILM Corporation, Tokyo Japan). The concentration of each sample was calculated using the Beer-Lambert Law (at 280nm) using the POLAR star Omega microplate reader (BMG Labtech, Offenburg, Germany).

Two-dimensional gel electrophoresis (2DE)

Triplicates (100µg) of each pooled sample were processed for reduction and alkylation then separated in the first dimension (Isoelectric focussing) using a 7cm non-linear pH = 3-10 IPG strip (Bio-Rad Laboratories, Hercules, California). IEF and 2D protein standards (Bio-Rad, Australia) with pI ranging from 4.45 to 9.6 were added to the sample mixture for identification following warping of gel images. IPG strips were then washed twice in IPG equilibration buffer (EB) with 2% DTT and then transferred (4°C at 150V) to a resolving gel (7x7cm SDS gels) and resolved (at 90V) until completion. 2DE gels were fixed, stained and de-stained using the Coomassie brilliant blue (CBB) [10; 24]. To reduce CBB detaching from proteins, gels were stored in 20% ammonium sulphate solution at room temperature before undergoing mass spectrometry. Gels were imaged using a FLA-9000 imager for CBB detection at 685nm [24].

Spot analysis on whole gels

Densitometry analysis of gel images was conducted using Delta 2D software (DECODON GmbH, Germany) with triplicate gels being used to determine significant differences ($p<0.05$) between experimental groups. Changes for upregulated and downregulated proteins were identified in comparing pre- and post-injury groups and pre- and post-minocycline in injured animals (CS vs. CM) (See Tables 2-6).

Spot picking and digestion

CBB stained spots of interest and were picked from the gels under sterile conditions using a pipette tip and processed for protein extraction [24]. Extracted proteins were digested with trypsin using previously published methods [24].

Criterion for spot selection and protein identification

Spots were identified on the basis of, (a): uniqueness, i.e. newly appearing spots after injury or minocycline, (b): of a 2-fold bidirectional (up or down regulated) change; and (c): from the top 10% of proteins across all groups. These spots were highlighted and selected for protein identification in the software. Uniqueness of proteins was identified by superimposing a stack of triplicate gels in one group with a triplicate stack of another group. Gel warping was used to align all spots as closely as possible. This was achieved by identifying the standard proteins with known pI and molecular weight throughout the separation and on the ladder (See left markers in gel from figure 5). Spots were identified and compared across triplicates to eliminate false positives. These proteins were then

categorised according to the direction of expression (up or down), in comparison of groups (e.g. injured vs minocycline treated), function and region of expression (e.g. membrane or soluble fraction), in bilateral (B), injured (I) or contralateral (C) sides.

Mass spectrometry analysis

Protein samples were analysed using mass spectrometry (Xevo G2-XS QTof, Waters) in the MS facility in Western Sydney University, Australia. The resulting spectra were analysed using MASCOT search engine (Matrix Science, USA). Extracted proteins were identified and categorised into functional groups using information from UNIPROT data (http://www.uniprot.org/). In the MS spectra, the protein with the highest peak was used as the identified protein. Proteins with MOWSE scores of >100 were reported.

Statistical analysis

Behaviour & Immuno-labelling

All results were expressed as mean ± standard error of the mean (SEM). The data from sensory testing was analysed using a repeated measures 2-way analysis of variance (ANOVA) with post hoc Dunnet's multiple comparison tests and the antibody staining was analysed using one-way ANOVA with Tukey's multiple comparison tests by means of GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Consistent with our prior study [57], a unilateral nerve crush resulted in a bilateral tactile and cold allodynia, an effect that was reversed by concomitant treatment with minocycline. In addition to preventing the allodynia, immuno-histological examination revealed that minocycline changes the pattern of microglia and astrocyte activation. Proteomic analysis not only confirmed these changes in glial cell function but also revealed a host of changes in protein expression that are shared by the ipsi- and contralateral dorsal horn.

Behaviour

Prior to nerve crush, the thresholds for allodynia (tactile and cold) and hyperalgesia (heat) were consistent across left or right forepaws and experimental groups. In all behaviour graphs, red dotted line shows injured rodent with saline treatment; blue dotted line shows injured rodent with minocycline treatment and solid black line shows naïve-uninjured rodent with saline treatment. Data is shown as mean ± SEM. As baseline tactile, cold and heat thresholds varied in each animal according to weight and gender, therefore for better representation of data, all data is shown as percentage change from the baseline set at 100%.

Cold allodynia

Prior to nerve crush, aversive responses to cooling were not observed even when plate temperatures were dropped to 4°C for 3 minutes. Whereas following nerve crush cold allodynia was observed when the plate temperature fell to 12°C. In contrast, following treatment with minocycline the plate temperature needed to be reduced to 8 $\pm$ 0.3^{o}C (median: 8, range: 6-8) to evoke cold allodynia (figure 1).

Tactile allodynia

As shown in figure 2A and B, a unilateral nerve crush (left, dashed red lines) evoked bilateral reductions in withdrawal thresholds that once established (day 2) persisted until day 7 (*left:* 70.2 $\pm$ 14%, *right:* 71.1 $\pm$7.7% P<0.001, F=6.8). In contrast, concomitant treatment with minocycline prevented the development of tactile allodynia, bilaterally at all time points with thresholds not significantly differing from their control values (e.g., Day 7, *left*: 111.0$\pm$16.0%, *right*: 85.7$\pm$5.1%, dashed blue lines).

In the absence of nerve crush (figure 2A, black solid line) repeated vehicle injections evoked a reduction in withdrawal threshold (day 7, *left*: 56.7$\pm$4.1%; *right*: 58.0.0$\pm$3.8%; P<0.001). This reduction in threshold evoked by repeated vehicle injections was significantly (P<0.001) attenuated by minocycline treatment (*left*: 73.1$\pm$8.6%; *right*: 73.0$\pm$7.4%, Figure 2E solid blue line).

Heat hyperalgesia

Following unilateral nerve crush (left), saline treated animals displayed a reduction in withdrawal on the injured side (*left*: 71.2$\pm$15.7%, P<0.05) that did not extend to the intact/ contralateral side (*right:* 105.7$\pm$8.3%, P>0.05). Treatment with minocycline prevented the

development of hyperalgesia (*left*: 113.0±24.6%, *right*: 109.7±6.5%) (See figure 2C and D). Moreover, the thresholds of minocycline treated group did not differ to those observed in the un-injured control rodents (figure 2F).

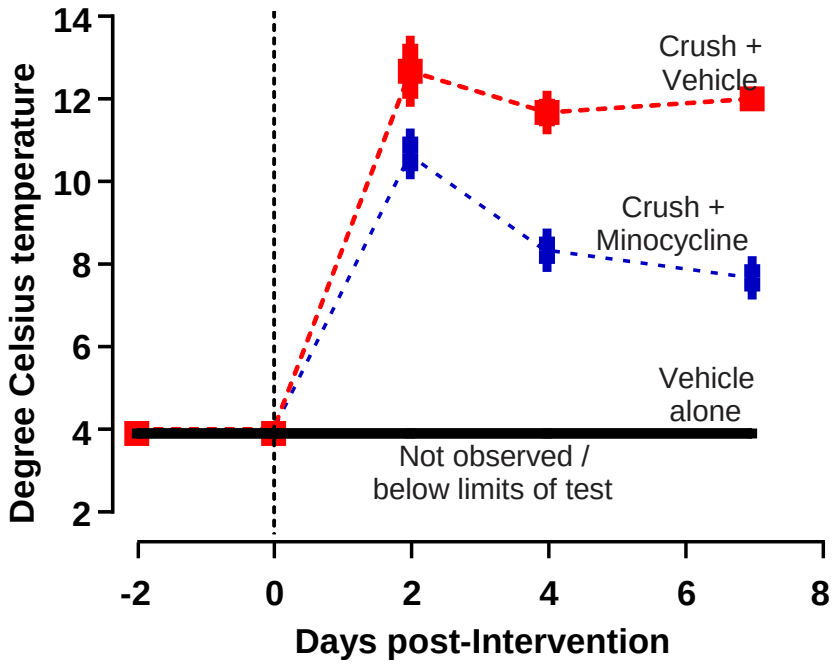

Figure 1: Temperatures at which cold allodynia was perceived post injuryfrom 4°C to 14°C.

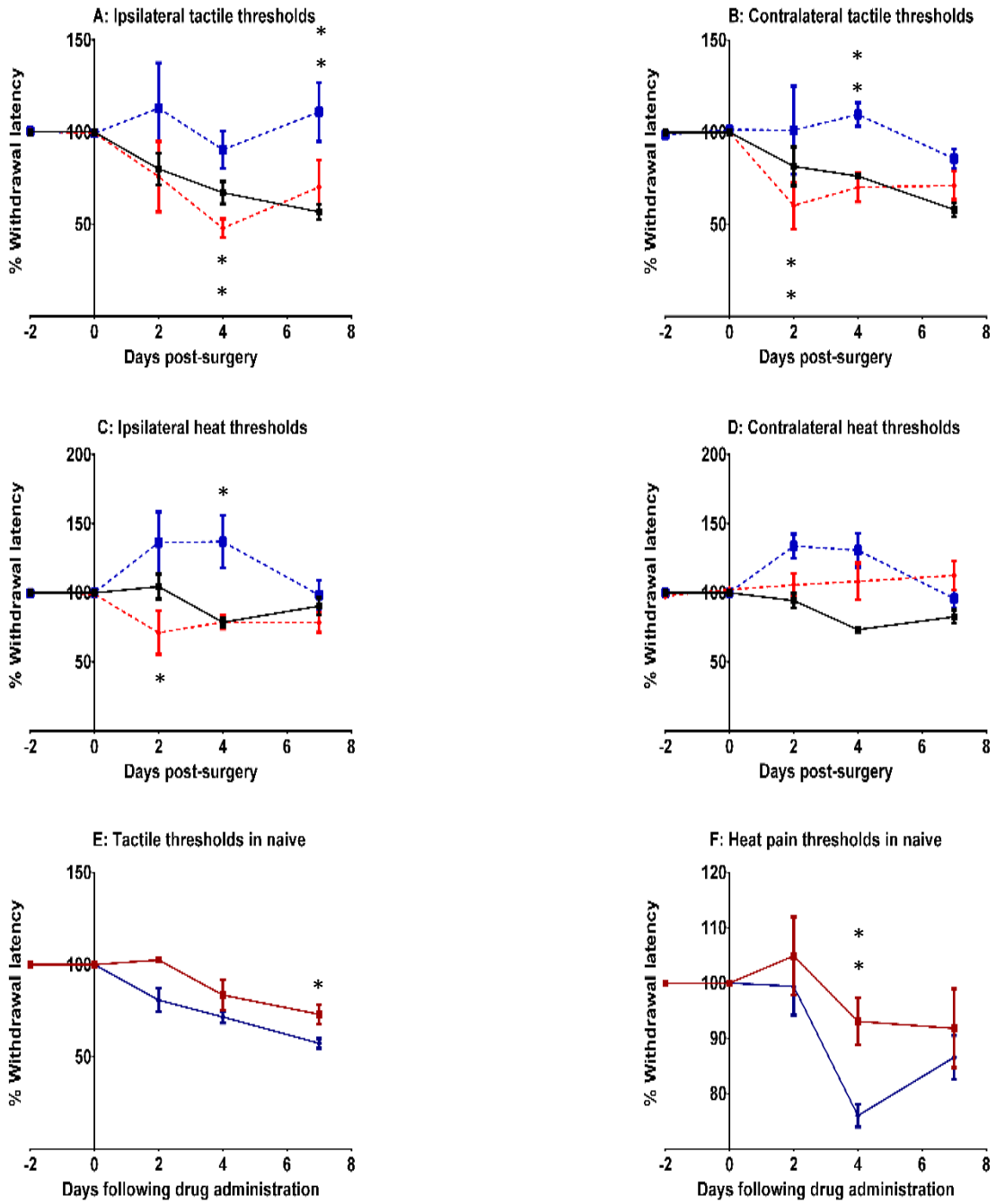

Figure 2: Behavioural thresholds for bilateral tactile allodynia on (A) ipsi- and (B) contra-lateral sides, unilateral heat hyperalgesia on (C) ipsi- and (D) contra-lateral sides, and tactile (E) and heat (F) thresholds in naïve animals in minocycline (solid red) and saline (solid blue) treated groups as a cumulative score of left and right sides as in sham (un-injured) groups, no significant differences were found in left and right sides. All data is shown as a percentage change from the baseline (100%).

Immunohistochemistry

Following the labelling of boundaries of lamina IIi using CGRP immunofluorescence as marker for the termination of small diameter nociceptive fibres, no differences in the intensity of CGRP staining were observed between experimental groups (control *v.* crush), left and right dorsal horns (see figure 4) and treatment (vehicle *v.* minocycline). However, this region was used to quantify astrocyte and microglial activity.

Microglia

Unilateral nerve crush evoked a bilateral increase in the number of $IBA1^+$ microglia (*left*: 140.7+6.9, *right:* 100.2+2.8) relative to that observed in uninjured saline treated animals (left, 75.6+1.5; right, 75.8+1.3; P<0.0001). Treatment with minocycline not only suppressed the bilateral increase in $IBA1^+$ evoked by nerve crush (left, 101.0+3.2; right, 68.2+2.9; P<0.0001) but also reduced the increased $IBA1^+$ cells observed following repeated saline was injections in uninjured animals (*left*, 42.8+2.0; *right*, 41.1+2.5; P<0.0001; See figure 3: label A, B and C; figure 4A).

Astrocytes

Unilateral nerve crush (left) evoked a bilateral increased GFAP fluorescence (*left:* 239.3±23.4 AU, *right*: 150.3±10.3 AU) compared to intact saline treated controls (*left*, 83.3±7.3; *right*, 78.7±11.3, P<0.001). Minocycline treatment of nerve crush animals produced little or no suppression of GFAP intensity on the side of injury (*left:* 202.7±30.4, P>0.05) and an increased contralateral on the side (right: 231.0±9.1, P<0.05) compared to saline treated animals (left: *right:* 150.3±10.3; P<0.05, See figure 3: label D, E and F; figure 4B).). In the absence of injury, GFAP intensity increased significantly post minocycline treatment (*left*, 126.3±15.7; right, 133.7±15.30) compared to saline treatment (*left*, 83.3±7.3; *right*, 78.7±11.3, P<0.05).

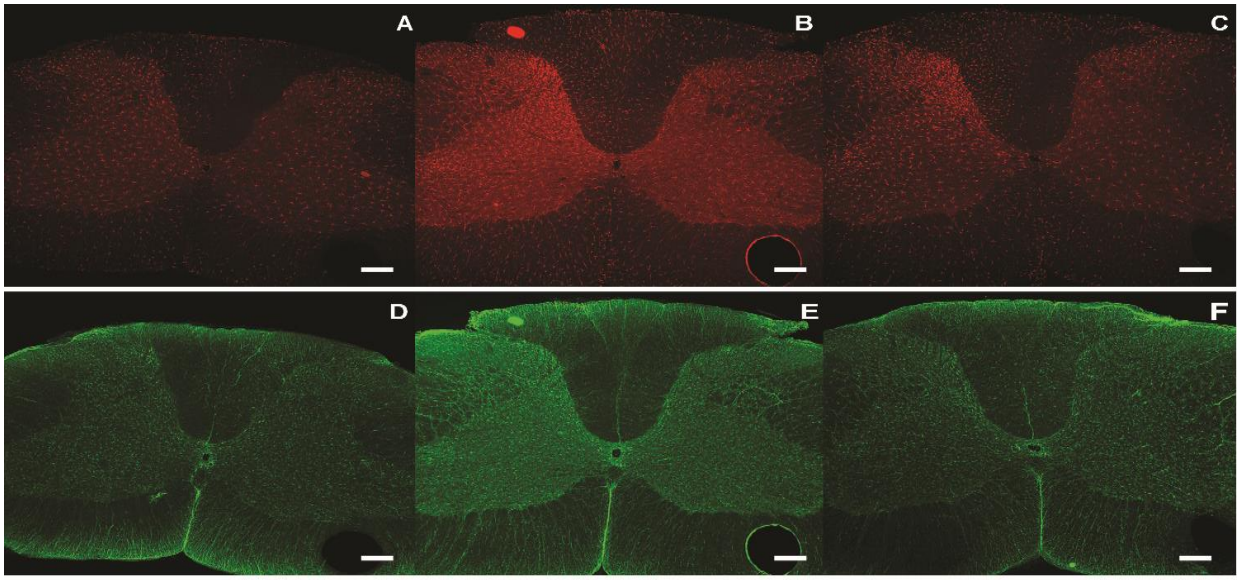

Figure 3: Immuno-labelling for microglial cells (red, top row) and astrocyte fluorescence intensity (green, bottom row) where C7 sections of labelled $IBA1^+$ cells of naïve (A), injured saline treated (B) and injured minocycline treated (C) as well as $GFAP^+$ cells of naïve (A), injured saline treated (B)

and injured minocycline treated (C) rodents are shown. Scale bar is 100µ. Bottom right hole suggests contralateral side.

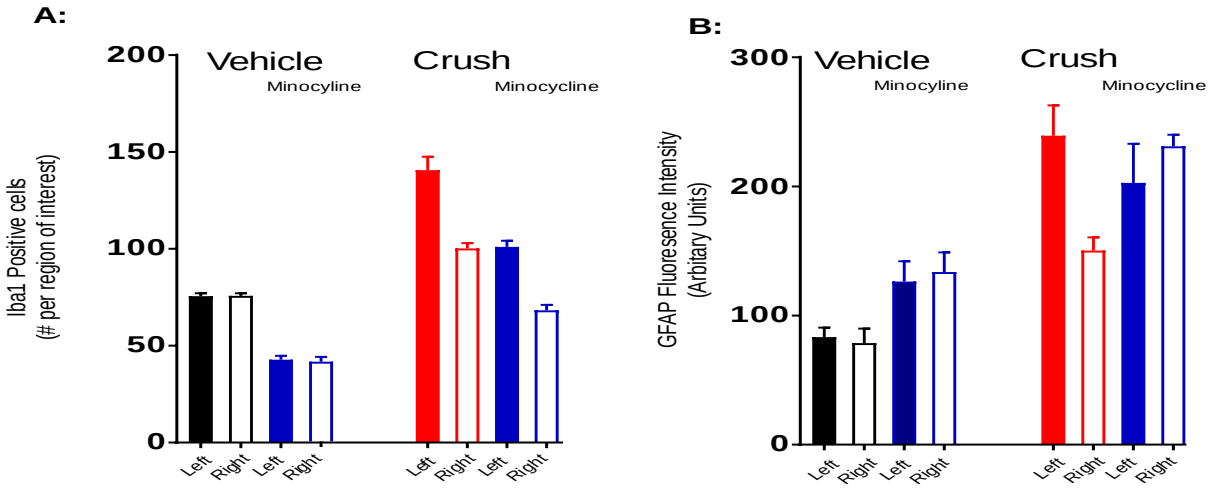

Figure 4: Quantification in laminae II_i of C7 sections of active microglial cell numbers (A) as well as astrocyte intensity staining (B). In A and B, black bars (n=2) on the left shows vehicle-saline treated naïve group and blue bars (n=2) on the right shows minocycline treated naïve group whereas red bars (n=2) on the left shows vehicle-saline treated injured group. Solid coloured (injured side) and outlined (contralateral side) bars of naïve (black), injured saline treated (blue) and injured minocycline treated (red) bars are shown. Data is from triplicate sections of each animal and shown as mean ± SEM.

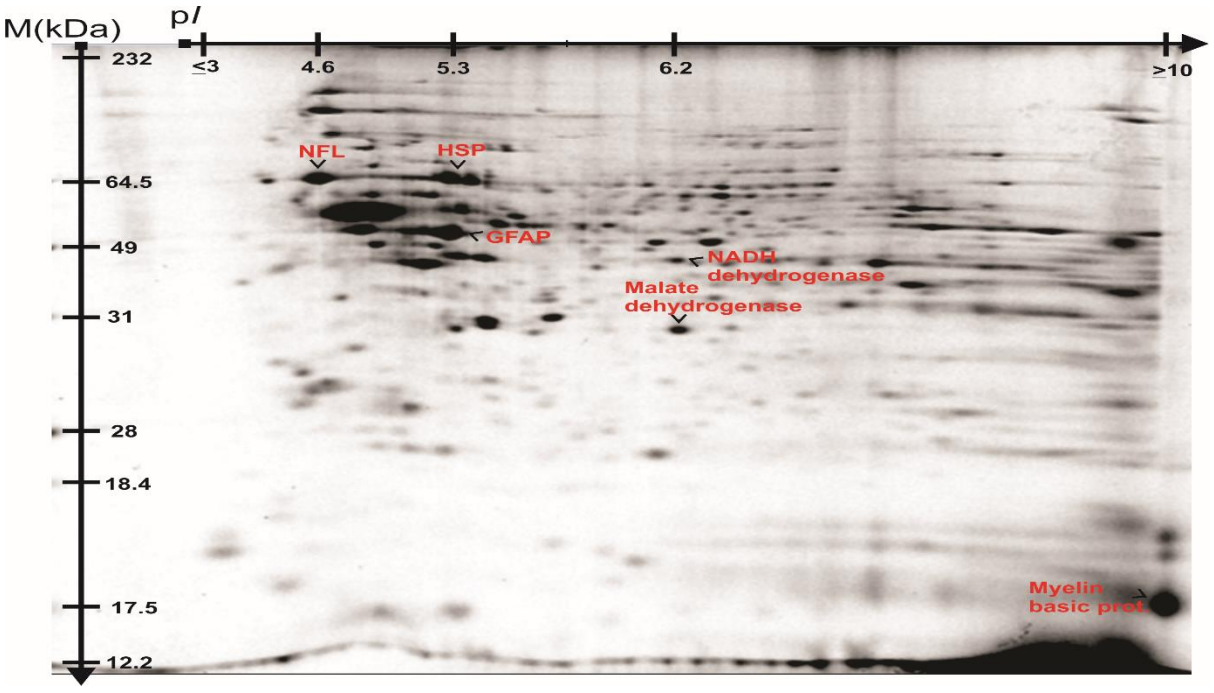

Figure 5: A sample gel of 7x7 cm with some of the commonly labelled spots stained with CBB across different groups. X axis represent the pI of range 3-10 and Y axis represents the mass or molecular weight in kDa

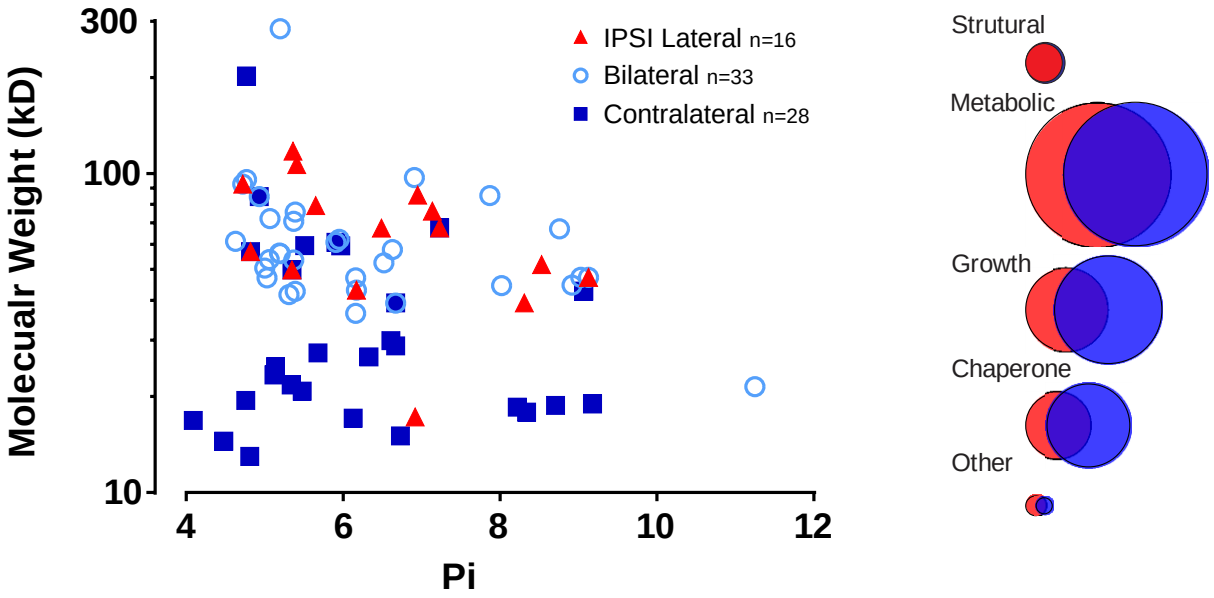

Figure 6: Left side represents a scatter plot of mass vs. pI index for all proteins found on injured, bilateral and ipsilateral sides. On the right, Venn diagrams show the overlap (bilaterally found) in functional groups of proteins, where red is proteins on injured side and blue shows proteins on contralateral side.

Proteomics

Densitometry analysis yielded over 1000 identifiable spots that were reproducible across technical replicates and experimental groups. Using the criterion stated above, this list was reduced to ~300 spots being excised from the gel (see figure 5), protein identification of which 77 proteins met the criteria of least 6 unique peptides. As shown in figure 6, when proteins were categorised on the basis on function five groups including structural (n=8), metabolic (n=28), growth and signalling (n=21), molecular chaperoning (n=16) and other uncategorised proteins (n=4) were defined. As shown in tables 2-6, these individual proteins were classified whether; they were expressed bilaterally (B), on the injured side (I) or contralateral side to injury (C) in membrane (M) and soluble (S) or both (M, S) domains of the dorsal horn. Number of matches is the number of total sequence matches with significant matches in brackets and the % seq. cov. is the percentage of matching sequence coverage for each protein. NA indicated either the absence of proteins or a below visibility expression in the gels.

Table 2: Structural proteins

No.	Protein name	Found	Side	Injury	Mino.	pI	Mass (kDa)	% Seq. Cov.	MOWSE Score	No. of matches	Function
1	Neurofilament light polypeptide	**M, S**	**B**	↑	↓	4.63	61.3	59	1610	61(43)	Maintainace of neuronal caliber
2	Actin, cytoplasmic	**S**	**B**	↑	↓	5.31	41.8	57	1078	32(25)	Contractile proteins but also implicated in LTP
3	Alpha-internexin	**M**	**B**	↑	↓	5.2	56.1	48	1036	37 (29)	Class-IV neuronal intermediate filament
4	Neurofilament medium polypeptide	**M**	**B**	↑	↓	4.77	95.7	35	679	40 (31)	Maintainace of neuronal caliber
5	Spectrin alpha chain, non-erythrocytic 1	**S**	**B**	↑	↓	5.2	284.5	16	601	39(20)	Structural protein, non-erythrocytic component is a biomarker for migraine and cancers
6	Vimentin	**M, S**	**B**	↑	↓	5.06	53.7	54	391	23(16)	Class-III intermediate filament protein
7	Peripherin	**M**	**B**	↑	↓	5.37	53.5	31	226	15(7)	Class-III neuronal intermediate filament protein
8	Microtubule-associated protein 2	**S**	**C**	↑	↓	4.77	202.3	4	136	7(3)	Structural microtubule implicated in neuropathic pain; Ligand for calcium binding proteins

Structural proteins

All structural proteins (n=8) displayed an upregulation following nerve injury (as compared to uninjured controls) that was reversed following minocycline treatment (see table 2). This pattern was repeated bilaterally except for one, microtubule-associated protein, which was only found on the contralateral side. Also, two proteins (neurofilament light and vimentin) were found on both membrane and in soluble components, while the number of proteins found only on membrane and only in soluble fraction was three each.

Peripherin is a specific marker for unmyelinated fibres with a - neurofilament (NF68) structural protein, which is important for axonal architecture and transport, and is co-localised with high threshold C nociceptive [8] as well as low threshold CLTMR primary afferents [22] that terminate in the superficial lamina in dorsal horn. Such afferent neurones expressing NF68 have been shown to make synaptic contact with neurons responsive to non-noxious stimulation (and expressing c-fos) suggesting possible role in the development of allodynia [66]. Vimentin, plays an important role in spatial translocation of phosphorylated MAP kinase (pERK), induction of apoptosis in injured nerves [49] and tactile allodynia [70].

Table 3: Metabolic enzymes

No.	Protein name	Found	Side	Injury	Mino.	pI	Mass (kDa)	% Seq. Cov.	MOWSE Score	No. of matches	Function
1	Alpha-enolase	M, S	B	↓	↑	6.16	47.1	78	3755	77 (68)	Non neuronal multifunctional glycolytic enzyme
2	Phosphoglycerate kinase 1	S	B	↑	↓	8.02	44.5	75	1574	56 (39)	Glycolytic enzyme
3	Fructose-bisphosphate aldolase	S	B	NA	↓	6.67	39.3	60	800	32(19)	Required in metabolic processes
4	Aspartate aminotransferase	S	B	NA	↓	9.13	47.3	39	627	26(19)	Implicated in metabolism of aspartate
5	L-lactate dehydrogenase	M	B	↑	↓	5.7	36.6	43	601	25(17)	Glycolytic enzyme
6	Pyruvate kinase PKM	S	B	↑	↓	6.63	57.8	44	532	30(19)	Glycolytic enzyme
7	Creatine kinase	S	B	↑	↓	5.39	42.7	37	479	10 (9)	Cellular metabolism mainly conversion of phosphate from ATP to creatine
8	ATP synthase subunit beta	M, S	B	↑	↓	5.19	56.3	31	272	11(10)	Makes ATP from ADP
9	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	M	B	NA	↑	8.76	67.1	13	208	10(6)	Enzyme required for cellular respiration
10	Gamma-enolase	M, S	B	↓	↑	5.03	47.1	35	178	15(6)	Neuronal glycolytic enzyme; neuroprotective and neurotrophic
11	Malate dehydrogenase, cytoplasmic	M, S	B	↓	NA	6.16	36.5	24	169	9(6)	Catalyzes the conversion of oxaloacetate and malate in cellular metabolism
12	Glycogen phosphorylase	M	B	↑	↓	6.91	97.2	7	104	8 (6)	Glycogen catabolism
13	NADH dehydrogenase [ubiquinone] iron-sulfur protein	M	B	↓	↑	6.52	52.5	10	100	6(4)	Electron transfer from NADH to ubiquinone in the respiratory chain
14	Acetyl-CoA acetyltransferase	S	B	↑	↓	8.92	44.7	5	100	2 (2)	Lipid metabolsim
15	Fructose-bisphosphate aldolase	M	I	NA	↓	8.31	39.3	56	707	30(18)	Required in metabolic processes
16	Aspartate aminotransferase	M	I	NA	↓	9.13	47.3	39	627	26(19)	Implicated in metabolism of aspartate
17	NADH-ubiquinone oxidoreductase	M	I	NA	↓	5.65	79.4	9	144	6 (5)	Mitochondrial respiratory chain enzyme
18	Phosphoglycerate mutase 1	S	C	↑	↓	6.67	28.8	73	1082	38(28)	Gluconeogenesis enzyme
19	Transketolase	S	I	↑	↓	7.23	67.6	34	684	30(18)	Enzyme of pentose phosphate pathway
20	L-lactate dehydrogenase	S	I	↑	↓	5.7	36.6	43	601	25(17)	Glycolytic enzyme
21	Peroxiredoxin-2	S	C	↑	NA	5.34	21.8	22	496	16(14)	Redox regulation of the cell
22	Citrate synthase	S	I	↓	↑	8.53	51.8	28	417	17 (12)	Enzyme in citric acid cycle
23	Transketolase	S	C	↓	↑	7.23	67.6	22	228	15(6)	Enzyme in pentose phosphate pathway
24	Glucose-6-phosphate 1-dehydrogenase	S	C	↑	↓	5.97	59.3	29	214	18(10)	Glycolytic enzyme
25	ATP-dependent 6-phosphofructokinase	S	I	↑	↓	6.95	85.7	11	106	8(4)	Glycolytic enzyme
26	Isocitrate dehydrogenase [NADP] subunit gamma 1,	S	C	↑	↓	9.07	42.8	12	154	6 (6)	NADH metabolic enzyme
27	Ubiquitin-conjugating enzyme E2 N	S	C	↓	↑	6.13	17.1	19	126	5(3)	ATP binding
28	NAD-dependent protein deacetylase sirtuin-2	S	C	↑	↓	6.67	39.3	22	105	7(5)	NAD dependent cellular metabolism

Metabolic proteins

Out of the total metabolic proteins (n=28), a large majority (n=14) were found bilaterally, and the rest were found on ipsi- (n=7) and contra- (n=7) lateral sides. As the majority of proteins from this group act as enzymes, 4 were found in both membrane and cytosolic fractions, whereas, 17 were exclusively found on the membrane and in cytosolic fractions each (see table 3).

Although, IBA1 was not detected within the parameters of the proteomic analysis (within the set criterion, e.g. top 10%), the profile of metabolic changes observed following nerve crush was indicative of progression to a high energy state. Namely, the upregulation enzymes within the glycolic pathways (ATP-dependent 6-phosphofructokinase; Glucose-6-phosphate 1-dehydrogenase; Phosphoglycerate mutase 1; Pyruvate kinase; L-lactate dehydrogenase) activity within TCA cycle (Acetyl-CoA acetyltransferase, Isocitrate dehydrogenase [NADP]), generation and use of ATP (Creatine kinase; ATP synthase subunit beta) and increased liberation of stored glucose (Glycogen phosphorylase). In contrast, the enzymes within the glycolytic pathway (neuronal and general enolase), mediating key step in the Calvin cycle (Transketolase) and electron transport chain function (NADH dehydrogenase; Ubiquitin-conjugating enzyme E2 N; Dihydrolipoyllysine-residue acetyltransferase) are downregulated during nerve injury.

In the presence of minocycline, the increased expression associated with increasing energy usage observed following nerve injury was prevented, as were the change in expression of those that are down regulated following nerve injury.

Growth & signalling proteins

Out of 21 total proteins in this category, 11 were found to be in the top changing list on the contralateral side, which suggests that these could be a part of major physiological changes, otherwise obscured on the injured side; whereas, 4 were found on the ipsilateral and the remaining 6 on the bilateral side (see table 4).

Replicating our immunohistochemistry results, the astrocyte marker glial fibrillary acidic protein (GFAP; MOWSE: 2360) was increased bilaterally following nerve injury. Note that GFAP is repeated twice in this list due to different change in expression in the contralateral side following minocycline treatment. This increase in contralateral expression of GFAP following injury is exacerbated with minocycline, whilst supressing the increased expression on the injured side observed following nerve injury. This latter pattern of increased expression following nerve injury that was abolished by minocycline was shared with 13 other proteins indicative of nerve injury (Myelin basic protein; Gamma-synuclein), neuronal outgrowth (Dihydropyrimidinase-related protein3 2; Cofilin-1) cell cycle regulation (Tubulin polymerization-promoting protein family member 3;S-phase kinase-associated protein 1) synaptic function (Aconitate hydratase; Annexin A6) and known proteins with roles in pain modulation (Calmodulin; Phosphatidylethanolamine-binding protein 1).

Table 4: Growth & Cell signalling proteins

No.	Protein name	Found	Side	Injury	Mino.	pI	Mass (kDa)	% Seq. Cov.	MOWSE Score	No. of matches	Function
1	Dihydropyrimidinase-related protein 2	M, S	B	↑	↓	5.95	62.2	52	1174	34(27)	Axonal outgrowth mediated by extracellular signals
2	Annexin A6	M	B	↑	↓	5.39	75.7	42	636	35 (22)	Exocytosis function
3	Aconitate hydratase	S	B	↑	↓	7.87	85.4	35	559	32(18)	Glutamate synthesis and lipogenesis
4	Rab GDP dissociation inhibitor	M, S	B	↑	↓	5	50.5	40	407	14 (12)	Regulates the GDP/GTP exchange reaction of most Rab proteins
5	Synaptic vesicle membrane protein VAT-1	M	B	↓	NA	6.17	43.1	20	176	6 (4)	Vesicular channel protein
6	Myelin basic protein	M	B	↑	↓	11.25	21.5	34	150	11(7)	Indicator of immune mediated demyelination
7	Glial fibrillary acidic protein	M	I	↑	↓	5.35	49.9	81	2360	80 (59)	Astrocyte communication; Indication of systemic inflammation
8	Glial fibrillary acidic protein	M	C	↑	↑	5.35	49.9	81	2360	80 (59)	Astrocyte communication; Indication of systemic inflammation
9	Syntaxin-binding protein 1	M	I	↓	↑	6.49	67.5	9	109	5(3)	Regulation of synaptic vesicle docking and fusion
10	Cofilin-1	S	C	↑	↓	8.22	18.5	64	1067	24 (18)	Implicated in neurite outgrowth via galanin receptor
11	Rho GDP-dissociation inhibitor 1	S	C	↓	↑	5.12	23.4	46	532	17(11)	Controls apoptotic process and axonogenesis
12	Calmodulin	S	C	↑	↓	4.09	16.8	51	507	14(11)	Calcium binding messenger protein implicated in neuropathic pain
13	Gamma-synuclein	S	C	↑	↓	4.81	13.0	69	301	13(8)	Implicated in neurodegenerative diseases and some neuropathies
14	Beta-synuclein	S	C	↑	↓	4.48	14.5	50	260	9(6)	Calcium ion binding; Marker of Alzheimer's disease
15	Phosphatidylethanolamine-binding protein 1	S	C	↑	↓	5.48	20.8	26	186	6(3)	Regulates MOR pathway
16	Tubulin polymerization-promoting protein family member 3	S	C	↑	↓	9.18	19.0	32	168	5 (4)	Cell proliferation
17	ProSAAS	S	C	↓	↑	5.68	27.4	26	164	8(4)	Inhibits prohormone convertase which regulates the endogenous opioidergic system
18	S-phase kinase-associated protein 1	S	C	↑	↓	4.76	19.5	30	142	5(3)	Involved in cell cycle progression and signal transduction
19	Ubiquitin carboxyl-terminal hydrolase isozyme	S	C	↓	↑	5.14	24.8	30	131	7(4)	Involved in MAPK pathway
20	Synaptic vesicle membrane protein VAT-1	S	I	↓	NA	6.17	43.1	20	176	6 (4)	Vesicular channel protein
21	Nucleoside diphosphate kinase B	S	I	↑	↓	6.92	17.3	51	152	10(6)	Ca^{2+}-activated K^{+} channel KCa3.1 is activated by this peptide through downstream of PI(3)P

In contrast, examination of those proteins that decreased following nerve injury and were reversed by minocycline treatment includes examples where the proteins normally supress cellular apoptosis (Rho GDP-dissociation inhibitor 1) and the activation of endogenous opioids (ProSAAS). This group also included a range of synaptic regulatory proteins (Syntaxin-binding protein 1; Synaptic vesicle membrane protein VAT-1) that play central roles in synaptic remodelling.

Table 5: Protein folding and chaperoning proteins

No.	Protein name	Found	Side	Injury	Mino.	pI	Mass (kDa)	% Seq. Cov.	MOWSE Score	No. of matches	Function
1	60 kDa heat shock protein	S	B	↑	↓	5.9	60.9	72	1996	50 (45)	Molecular chaperone of the heat shock family
2	Heat shock protein HSP 90	S	B	↑	↓	4.9	84.8	35	809	26 (19)	Molecular chaperone of the heat shock family
3	Heat shock cognate 70, 71 kDa protein	M, S	B	↑	↓	5.4	70.8	45	790	34(24)	Signal transduction and protein folding
4	78 kDa glucose-regulated protein	M	B	NA	↑	5.1	72.3	31	172	16(10)	Molecular chaperone regulated by glucose
5	Endoplasmin	M	B	↑	↓	4.7	92.7	12	108	8(5)	Chaperone in endoplasmic reticulum
6	60 kDa heat shock protein	M	C	↑	↓	5.9	60.9	72	1996	50 (45)	Molecular chaperone of the heat shock family
7	Heat shock protein HSP 90	M	C	↑	↓	4.9	84.8	35	809	26 (19)	Molecular chaperone of the heat shock family
8	Protein disulfide-isomerase	M, S	I	↑	NA	4.8	56.9	42	488	20 (14)	Protein folding; electron transport
9	Protein disulfide-isomerase	M	C	↑	NA	4.8	56.9	42	488	20 (14)	Protein folding; electron transport
10	Endoplasmin	S	I	↑	↓	4.7	92.7	39	848	43(26)	Chaperone in endoplasmic reticulum
11	Serotransferrin	S	I	↑	↓	7.1	76.3	14	193	11(7)	Iron binding transport protein
12	Peptidyl-prolyl cis-trans isomerase A	S	C	↑	↓	8.3	17.9	74	653	26 (22)	Protein folding catalyst
13	Sorting nexin-3	S	C	↑	↓	8.7	18.8	40	153	8 (6)	Role in transport between cellular compartments
14	Fatty acid-binding protein	S	C	↑	↓	6.7	15.1	47	204	8(5)	Lipid chaperons
15	T-complex protein 1 subunit epsilon	S	C	↓	↑	5.5	59.5	16	187	8 (6)	ATP binding molecular chaperone
16	LIM and SH3 domain protein 1	S	C	↓	↑	6.6	30.0	30	102	8(4)	Ion transmembrane transport activity

Proteins as molecular chaperons

Similar to the growth and signalling category, a large majority of proteins (8) were expressed on the contralateral side. From the total of 16 proteins, 5 were expressed bilaterally and 3 were found only on the injured side. In addition to that, 9 were found only in the soluble fraction, 5 on the membrane and 2 were found in both fractions (see table 5).

This category, that functioned as molecular chaperons as well as for protein folding, 13 upregulated proteins associated with roles in iron binding (Serotransferrin), protein folding (Protein disulfide-isomeras; Peptidyl-prolyl cis-trans isomerase A; Protein disulfide-isomerase), Heashock (60, 90 kDa) and transport between compartments (Sorting nexin-3; Fatty acid-binding protein; Endoplasmin) were inhibited by minocycline treatment. The 3 proteins involved in the folding of actin and tubulin (T-complex protein 1 subunit epsilon) and (LIM and SH3 domain protein 1) and chaperoning (78 kDa glucose-regulated protein) were down regulated following injury and reversed following minocycline treatment.

All other proteins

Lastly, from a total of 4, membrane (n=3) and soluble (n=1), proteins were found that expressed ipsilaterally (n=2), contralaterally (n=1) and bilaterally (n=1) that were categorised functionally in none of the groups above (see table 6). The functions of these proteins range from biomarker of inflammatory conditions to enzymatic properties in dorsal horn cells.

Table 6: All other proteins

No.	Protein name	Found	Side	Injury	Mino.	pI	Mass (kDa)	% Seq. Cov.	MOWSE Score	No. of matches	Function
1	2',3'-cyclic-nucleotide 3'-phosphodiesterase	**M**	**B**	↑	↑	9.03	47.2	20	237	10(5)	An oligodendrocyte schwann cell and myelin-associated enzyme
2	Alanine--tRNA ligase, cytoplasmic	**S**	**I**	↑	↓	5.41	106.7	32	494	25 (17)	Implicated in inflammatory disorders
3	Ubiquitin-like modifier-activating enzyme 1	**S**	**I**	↑	↓	5.36	117.7	14	256	12 (9)	Biomarker and drug target for inflammatory diseases such as dermatomyositis
4	Glutathione S-transferase	**S**	**C**	↑	↓	6.33	26.6	32	293	11(8)	Phase II detoxification enzymes

Discussion

The majority of research in chronic pain focuses on the region of damage and its impact on the projection to the spinal cord namely the area of dorsal horn corresponding to the injury. Here, we have shown for the first time that bilateral sensory perturbations are associated with distinct proteomic changes that extend bilaterally raising serious questions as to whether contralateral measurements can serve as a control for pain studies. Understanding the complexities of these changes may well be central to our understanding of the neuropathic pain behaviour with symptoms such as bilateral tactile and cold allodynia.

By using minocycline pre-treatment to modulate bilateral allodynia post-unilateral median nerve injury, only those proteins associated with the change in condition were analysed as a function of change in behaviour. Glial associated changes on both sides of the dorsal horn were also observed to confirm the surgery and the impact of minocycline modulated behaviour. Since minocycline is a microglial inhibitor, as expected, the sensory behaviour correlated with microglial activation and proliferation. Furthermore, this cascade of inhibition also affected the astrocyte activity. As discussed earlier, allodynia can be mediated by A-LTMR and C-LTMR both [39; 42; 47; 53], these glial changes were specifically quantified in the lamina II_i as the region is known to have key importance in C-fibre mediating allodynia [47; 55]. Furthermore, the direction of change in proteins observed bilaterally as a function of sensory modulation due to minocycline implies these proteins may have a key role in bilateral hypersensitivity (i.e. tactile and cold allodynia).

Understanding allodynia: from the periphery to dorsal horn

Historically, cutaneous allodynia has been attributed to myelinated low-threshold A fibres [11; 13; 38; 61; 62]. This attribution has been reinforced by observation that blockade of myelinated A fibres abolished allodynia [12; 26; 31; 61]. In many studies, this link was characterised using a heat/capsaicin model where a circumscribed allodynia [61] is dependent on ongoing pain [4; 26; 31; 33]. In contrast to this 'canonical' view, correlative psychophysics [40-42] and microneurography [15] revealed that cutaneous allodynia can persist following blockade of myelinated fibres, suggesting a role for low threshold C afferents. Critically, these experiments demonstrated that allodynia can be evoked acutely (within minutes) based on C fibre input – indicating that allodynia is not solely dependent on the sprouting of large diameter inputs into the superficial lamina of the dorsal horn as suggested by Woolf and his colleagues [67; 68]. Whether large diameter fibres are the sole contributor has been questioned by the demonstration that small diameter fibres, including those that project to LII_i, undergo a phenotypic change characterised by an up-regulation of the binding site for CTB, so that almost all small neurons are labelled [5; 60].

In this study, sensory testing reproduced our earlier observation that unilateral nerve injury induced bilateral tactile and cold allodynia [57]. Whether, this is directly attributable to one

class of afferent fibre or the convergence of many, cannot be resolved by the techniques in this paper. However, the emergence of a tactile-cold allodynia phenotype that matches the response properties of C-LTMRs which are known to respond to touch and innocuous cooling, raises the distinct possibility that these fibres, through their projection to LII_i, may also contribute to the allodynia observed in this paper. Furthermore, this builds on recent work in rodents, demonstrating that, tactile and cold allodynia is dependent on the CLTMRs in rat and mice [17; 18; 22; 35] and our confirmation of this finding in humans [39; 53] reinforces the role for low threshold C fibres in the detection of innocuous touch and cooling that is regarded as painful following injury.

The role of minocycline in tactile and cold allodynia

Concomitant minocycline treatment prevented not only the development of cutaneous allodynia, but also, microglial cell activation and modulated the metabolic changes observed in our proteomic analysis. Here, the injury-induced bilateral microglial activation by minocycline was quantified in LII_i, as area that receives inputs from low threshold C afferents (such as C-LTMRs) that are glutamatergic (excitatory) fibres that express $VGLUT3^+$ [55] and inhibitory molecules including $GINIP^+$ [23] and TAFA4 [17]. In addition, LII_i receives inputs from interneurons arising in deeper lamina (III-V) that are activated by myelinated large fibre tactile inputs. This convergence in LII_i means that low threshold inputs from small and large diameter afferents can gain access to nociceptive specific neurones in lamina I depending upon the gating function within LII_i. Consequently, this region is anatomically and chemically equipped to underpin the emergence of allodynia. In addition to the tactile inputs, LII_i is accoutred with the modulatory circuitry of interneurons, microglia and astrocyte cell bodies and their processes that regulates metabolic, growth and signalling processes. The observed increase in microglial activity coincided with the emergence of bilateral allodynia – an effect that was reversed by concomitant treatment with minocycline.

A recent study showed that microglia are not required for mechanical pain hypersensitivity in female mice and used adaptive immune cells, likely T lymphocytes instead [58]. However, in our species of rats, there was no difference in behaviour for pain sensitivity between males and females and hence, the distinction in gender was not made while allocating these rats, i.e. at any given time, there were both males and females in one group.

Contralateral side of astrocytic modulation

Like microglia, nerve injury increased GFAP expression in astrocytes bilaterally, whereas concomitant treatment with minocycline had varying effects on GFAP immuno-labelling: exacerbated the increase in contralateral expression observed following nerve injury and; had no effect on stabilising the expression in the ipsilateral dorsal horn. This variation in effect may, at least in part, be the result of regional variations in the relatively small region (LII_i) of analysis. However, proteomic analysis that independently sampled the entirety (i.e. homogenates of all dorsal horn lamina and associate white matter) of the individual ipsi- and contra-lateral dorsal horn confirmed the bilateral increase in GFAP expression evoked

by nerve injury demonstrated that, minocycline treatment exacerbated the increase in contralateral expression observed following nerve injury, whilst suppressing the increased expression observed following nerve injury. A reason for this enhanced activation on the contralateral side could be the glycolysis, i.e. glucose utilization and lactate production, in astrocytes [3]. It is reported that glutamate, an indicator of neuronal excitability, is related to the stimulation of glycolysis [48]. This removal of glutamate coupled with glucose utilisation could be a process of ongoing restoration following injury. On the contralateral side, in the absence of injury but with altered sensitivity, astrocytes may have found an alternate way to combat glutamatergic mediated neuronal excitability that resulted in an increased functioned expression

In addition our results validated this GFAP expression, proteomic analysis revealed a host of proteins expressed on neurons that were found in ipsi- and contra-lateral samples (table 2-6). The densitometry analysis of the 2D gels revealed over 1000 individual spots, when sorted on the basis of those spots that evoked a reproducible change (>2 fold) that was modulated by minocycline revealed a subset of proteins spanning structural, metabolic, signalling and chaperoning functions. Of these, the top 10% were sampled yielding ~80 confirmed mass-spec identities.

The role of glia in metabolic pathways post-injury

Consistent with glia's role in the utilisation of oxygen and glucose to provide the CNS with a continuous supply of ATP, following nerve injury the changes in metabolism were dominated by upregulation enzymes within the glycolic pathways, generation and use of ATP and increased liberation of stored glucose. Such changes not only expose the cells to increased oxidative stress but also support the macrophage-like properties of glia. For example, Aldose Reductase (AR) mediates the metabolism of glucose and has shown to be upregulated in many conditions including neuropathy[20; 32], effects that are diminished by AR inhibition [32]. Likewise, as shown in this paper, work in isolated macrophages has shown that LPS induction of AR is supressed by minocycline [27]. Given that NADH is a co-factor for AR function, a proposed mechanism of action of minocycline is to act as an NADH mimetic and inhibit enzymatic function of poly-(ADP) ribose polymerase [2].

Astrocytes and ATP

Using optogenetic stimulation of astrocytes, a recent study demonstrated that ATP release from astrocytes can lead to central sensitisation and that the depletion of microglial cells post sensitisation attenuated pain hypersensitivity [43]. It was hypothesised that the astrocytic release of ATP, which breaks down to adenosine and stimulates A1 receptors on inhibitory interneurons to initiate hypersensitivity. ATP also activates the purinergic receptors, which are expressed on microglia, astrocytes and neurons in the spinal cord [63; 64] and are associated with the release of pro-inflammatory mediators and pain processing [19].

Astrocytes to GABAergic inhibitory systems

In the same optogenetic study discussed above, the stimulation of astrocytes induced central sensitisation produced greater mechanical allodynia than thermal hyperalgesia [43], a result consistent with this study where bilateral mechanical and cold allodynia was consistently reproduced as opposed to the more limited unilateral heat hyperalgesia. Additionally, their electrophysiology data also showed that ATP released from activated astrocytes inhibited GABAergic inhibitory interneurons, via A1-receptor-dependent pathway, to produce hypersensitivity [43]. It is known that GABAergic and glycinergic inhibitory systems contribute to mechanical allodynia but not thermal hyperalgesia [50]. Moreover, the GABAergic system have been associated with C-LTMR mediated mechanical allodynia following injury and GINIP, a protein that distinctly labels these afferents [23]. Furthermore, in GINIP deficient mice, the presynaptic inhibition of LII_i interneurons (the region of glial quantification in this study) was defective with reduced inhibition of voltage activated calcium (Ca^{2+}) channels [23] such as $CaV_{3.2}$ and $CaV_{3.3}$. These Ca^{2+} channels also selectively label C-LTMRs in mice [22; 52] and its antagonist blocks CT mediated cold allodynia in humans [53]. A universal pathway in glial cells is ATP evoked Ca^{2+} signalling in response to mechanical stimulation and excitatory glutamate. A key pathway to pain modulation is phosphorylation of pain receptor and ion channels such as $CaV_{3.2}$ or its associated proteins on the cell surface or in the cytosol that regulate its function that functionally changes the expression of these channels on the primary afferents.

Therefore, the modulation of glia in rodents with mechanical and cold allodynia in the context of inhibitory systems and C afferents, and its dorsal horn protein changes holds potential novel findings that could target pain hypersensitivity post-injury.

Innate immunity and injury induced hypersensitivity

Heat shock protein 60 or HSP 60 expression has been shown to act as a warning for the immune system and to initiate a strong pro-inflammatory response in the immune system[28]. The upregulation of both HSP60 and HSP70 in response to lipopolysaccharide, CD14 receptor, has been well documented [9; 46]; however, the two HSPs identified here showed different patterns of expression, HSP60 was downregulated in response to LPS whereas HSP71 was upregulated in response to LPS.

Numerous papers have demonstrated an upregulation of Heat shock proteins (HSPs) following spinal cord injury, nerve ligation and crush [16; 25; 29]. These HSPs act as ligands for toll-like receptors [44; 65; 69] that are a known link between innate immunity and injury and are upregulated in injury induced allodynia, microglial activation and functional recovery post-injury [7; 30; 59]. In our study the upregulation of HSPs observed following injury was suppressed by minocycline. While it cannot be determined whether this was due to a direct action of minocycline on HSPs or secondary to decreased structural damage, as indicated by markers of nerve injury (myelin basic protein; gamma-synuclein), or a suppression of HSP-induced apoptosis, we did observe a reciprocal regulation of protein

controlling cell cycle (upregulated: Tubulin polymerization-promoting protein family member 3;S-phase kinase-associated protein 1) and proteins that normally supress cellular apoptosis (down regulated: Rho GDP-dissociation inhibitor 1) following nerve injury that was reversed by minocycline.

Growth and functional restoration post-injury

In addition to the shift towards cellular apoptosis, the upregulation of proteins that stimulate neuronal outgrowth (Dihydropyrimidinase-related protein3 2; Cofilin-10) and synaptic function (Aconitate hydratase; Annexin A6) and down regulation of synaptic regulatory proteins (Syntaxin-binding protein 1; Synaptic vesicle membrane protein VAT-1) provides a mechanism for remodelling of neuronal micro-architecture and function following nerve injury. The capacity of minocycline to provide reciprocal modulation (i.e., supress the up and down regulations induced by nerve injury) provides a powerful mechanism by which the normal segregation of innocuous and noxious inputs can be preserved following nerve injury. Furthermore, the capacity of minocycline to induce a reciprocal modulation of the opposing mechanism involved in pain processing adds to the preservation of normal function. That is, the suppression of nerve injury evoked increase in expression of proteins associated with pain induction (Calmodulin; Phosphatidyl-ethanolamine-binding protein 1) and decreasing expression of proteins responsible for the activation of endogenous opioids (ProSAAS).

In this study, this category of proteins also included a range of synaptic regulatory proteins (Syntaxin-binding protein 1; Synaptic vesicle membrane protein VAT-1), cell cycle regulation (Tubulin polymerization-promoting protein family member 3; S-phase kinase-associated protein 1) synaptic function (Aconitate hydratase; Annexin A6) and known roles in pain modulation (Calmodulin; Phosphatidylethanolamine-binding protein 1).

In contrast, examination of those proteins that decreased following nerve injury and were reversed by minocycline treatment includes examples where the proteins normally suppress cellular apoptosis (Rho GDP-dissociation inhibitor 1) and the activation of endogenous opioids (ProSAAS). This category also included a range of synaptic regulatory proteins (Syntaxin-binding protein 1; Synaptic vesicle membrane protein VAT-1).

Conclusion

We have shown the role of microglial inhibition and astrocyte activation in the context of bilaterally extended mechanical and cold allodynia following a unilateral nerve injury using dorsal horn protein changes. The intra- and extra-cellular pathways related to the protein changes mentioned in this study involve interactions that include signalling cascades to increase and maintain the excitability of the neurons that underpin the emergence of allodynia. These results shed new light on existing mechanisms and highlight novel protein targets that bridge systemic immunity, metabolism and pain processing pathways. These results can be an important groundwork to view known interactions in a collective system as opposed to studying receptors and molecules individually in ex vivo. More importantly, the processes with injury and minocycline treatment are consistent with earlier view of changes in structure, metabolic activity, growth and modulatory signalling as well as transport proteins.

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Paper III

PLOS ONE

RESEARCH ARTICLE

Psychophysical Investigations into the Role of Low-Threshold C Fibres in Non-Painful Affective Processing and Pain Modulation

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Abstract

We recently showed that C low-threshold mechanoreceptors (CLTMRs) contribute to *touch-evoked pain* (allodynia) during experimental muscle pain. Conversely, in absence of ongoing pain, the activation of CLTMRs has been shown to correlate with a diffuse sensation of *pleasant touch*. In this study, we evaluated (1) the primary afferent fibre types contributing to positive (pleasant) and negative (unpleasant) affective touch and (2) the effects of tactile stimuli on tonic muscle pain by varying affective attributes and frequency parameters. Psychophysical observations were made in 10 healthy participants. Two types of test stimuli were applied: stroking stimulus using velvet or sandpaper at speeds of 0.1, 1.0 and 10.0 cm/s; focal vibrotactile stimulus at low (20 Hz) or high (200 Hz) frequency. These stimuli were applied in the normal condition (*i.e.* no experimental pain) and following the induction of muscle pain by infusing hypertonic saline (5%) into the tibialis anterior muscle. These observations were repeated following the conduction block of myelinated fibres by compression of sciatic nerve. In absence of muscle pain, all participants reliably linked velvet-stroking to pleasantness and sandpaper-stroking to unpleasantness (no pain). Likewise, low-frequency vibration was linked to pleasantness and high-frequency vibration to unpleasantness. During muscle pain, the application of previously pleasant stimuli resulted in overall pain relief, whereas the application of previously unpleasant stimuli resulted in overall pain intensification. These effects were significant, reproducible and persisted following the blockade of myelinated fibres. Taken together, these findings suggest the role of low-threshold C fibres in affective and pain processing. Furthermore, these observations suggest that temporal coding need not be limited to discriminative aspects of tactile processing, but may contribute to affective attributes, which in turn predispose individual responses towards excitatory or inhibitory modulation of pain.

Introduction

It is widely appreciated that large myelinated mechano-afferents subserve the sensory-discriminative facet of touch, which includes pressure, vibration/texture, stretch and movement of hair follicles. In addition to the well-studied aspects of discriminative touch, there exists a distinct and independently variable affective quality of tactile sensation that contributes to our emotional response to touch [1,2]. However, it is the more commonly recognised affective dimensions of pain that have been the main focus of research with affective touch having drawn relatively little interest over the years [3–5]. Affect, an inherently subjective process, can manifest in different ways across individuals, even amongst those with previously similar sensory experiences. The inter-individual differences could be attributed to the manner in which individuals perceive a particular sensation, and whether it enhances or diminishes the link with other cognitive (e.g. fear, tension, etc.) and associated autonomic events that could be shaped by past experiences and perceived implications of an existing event [6,7].

Microneurography studies have demonstrated a class of C low-threshold mechanoreceptors (CLTMRs) in the human skin (N.B. In this paper, the abbreviation 'CLTMRs' refers to the C low-threshold mechanoreceptors found in a myriad of species, including *C-tactile fibres* in humans). This afferent class responds to non-noxious touch with a predilection for slow-moving, low-force, stroking stimuli such as gentle brushing [8–10]. It was shown that CLTMRs exhibit an inverted U-shaped (negative quadratic) response curve to single strokes of graded brushing velocities with peak discharge occurring at 1.0–10.0 cm/s. Conversely, the activity in myelinated afferents exhibited a linear relationship with stimulus velocity. Interestingly, the subjective ratings of perceived pleasantness also followed an inverted U-shaped pattern in relation to brushing velocity. Based on a correlation between neural discharge (impulse/s) and perception, it was concluded that CLTMRs mediate positive affective or pleasant touch [11]. However, it is also noteworthy that the use of a scale with the endpoints 'unpleasant' (-10) and 'pleasant' (+10) meant that the subjects reported low-velocity brushing (0.1 cm/s) in the negative/unpleasant range [11]. Whether this effect could be attributed to large- or small-fibre function, or the need for temporal summation, is a matter for conjecture. However, such bimodal association has been reinforced in recent work demonstrating that variation in the temperature of skin-stroking outside the thermal neutral zone can decrease the positive affect and enhance the negative affect [11,12]. In our previous psychophysical studies, we tested the effects of gentle brushing at CLTMR-optimal speeds of 1.0 and 3.0 cm/s–using the same robotic device for brushing as used in the aforementioned pleasant-touch work (and in current study)–on a clearly perceptual, stable level of ongoing muscle pain. We found that the otherwise non-painful brushing stimuli–applied for 30 s–generated a stimulus-locked exacerbation of the overall pain intensity, i.e. allodynia. This effect was elicited whether the myelinated fibres were conducting or not, thereby suggesting a role of low-threshold C fibres in allodynia [13,14]. However, other studies have hypothesised a rather indirect role of CLTMRs in pain processing by way of the malfunction of the pleasant-touch system [15].

In the current study we explored the following questions: What types of peripheral afferent fibres mediate pleasant and unpleasant tactile sensations? What are the effects of normally pleasant and unpleasant stimuli on tonic muscle pain? We opted for two types of test stimuli with different spatio-temporal properties: stroking stimulus using velvet or sandpaper at slow to moderate speeds; focal vibrotactile stimulus at low or high frequency. The interplay between affective processing and pain modulation was tested by applying both positive and negative affective stimuli during ongoing muscle pain, which was induced, and maintained, by a continuous infusion of hypertonic saline. All interactions were retested following a conduction blockade of myelinated fibres by compression. It was hypothesised that, following the induction of

muscle pain, the *unpleasant* stimuli would manifest as *allodynia* while the *pleasant* stimuli would produce *analgesia*. Furthermore, it was hypothesised that the affective dichotomy and its subsequent modulatory effects on pain would remain preserved following the blockade of myelinated fibres.

Methods

Healthy human subjects (n = 10; 3 females and 7 males) with no known musculoskeletal disorders or neuropathies participated in this study. This study was approved by the Human Research Ethics Committee (approval number: H9190) of the University of Western Sydney and complied with the principles of the revised Declaration of Helsinki. Informed written consent was obtained from each subject before commencing the experiment. All subjects were naïve to the aims and objectives of the study. In all experiments, subjects sat comfortably on a chair with both legs supported and extended for easier access to the tibialis anterior (TA) muscle. The muscle was palpated during active inversion of the foot and dorsiflexion of the ankle joint for identification of its anatomical boundaries.

In order to determine the contribution of different fibre classes to the affective (pleasant-neutral-unpleasant) components of tactile perception (***Experiment I***) and subsequently to pain modulation (***Experiment 2***), two types of stimuli–***stroking stimuli*** of varying textures (velvet and sandpaper) and focal ***sinusoidal*** vibration (low- and high-frequency)–were applied in both experiments while all nerve fibres were intact and following the blockade (***Compression***) of myelinated afferents. As with our earlier work [13], a two-compartment model was adopted: pain was induced in the TA muscle, and tactile/affective stimuli were applied to the overlying skin. This was aimed at avoiding any ambiguity as to whether the change in pain perception during concurrent innocuous stimulation reflected an altered responsiveness of cutaneous nociceptors or an altered integration of inputs at the central level, i.e. peripheral or central sensitisation.

Stroking stimuli were applied using a robotic device known as Rotary Tactile Stimulator (RTS: Dancer Design, UK). This device has been used extensively in CLTMR research to study their responsiveness to graded brushing velocities. For details, see [11]. In the current study, sandpaper (300 Grit) and velvet fabric–length 4 cm and width 3 cm–were attached to the manipulandum that swept across the skin surface (overlying TA) along a proximo-distal axis. These stimuli were applied at stroking velocities of 0.1, 1.0 and 10.0 cm/s, and a calibrated normal force of 0.4 N. Stroking velocities of 1.0 and 10.0 cm/s were chosen because they have been shown to produce a pronounced discharge in the CLTMRs; an effect that correlated with touch pleasantness ratings. Conversely, 0.1 cm/s was chosen because of its apparent capacity to elicit an unpleasant sensation [11]. Given the constraints of time following the induction of muscle pain, it was not feasible to test more than three stroking velocities. For the same reason, only a single stroke was applied per stimulus. Each stimulus combination was tested in triplicates, and applied in a random order to the same region of skin.

Sinusoidal vibration was applied to the skin overlying the TA using a circular Perspex (Plexiglas) probe with a 4-mm diameter tip. The probe was positioned perpendicular to the skin surface $\sim$15 cm distal to the tibial tuberosity and $\sim$1.5 cm lateral to the anterior border of tibia [13]. The probe was attached to a feedback-controlled mechanical stimulator. The frequency (20 and 200 Hz) and amplitude (200 μm) parameters of the stimuli have been previously used to study the discriminative aspects (localisation, intensity and frequency) of the classical, large-fibre-mediated tactile sensations but have not been systematically used to quantify affective responses [16–18]. However, based on our previous work in the cat, it is deducible that a stimulus of this sort can also activate low-threshold small-diameter fibres, including C fibres [19].

Likewise, the stimulus duration (30 s) was selected on the basis of previous observations where the onset of allodynic responses to C-fibre activation was delayed by ~10–15 s [13,14]. Low (20 Hz) and high (200 Hz) frequency stimuli were presented three times in a randomised order.

Experiment 1: Affective responses

In *Experiment 1*, the affective qualities of stroking and vibrotactile stimuli were tested in 10 subjects using a Positive Affect and Negative Affect Scale **(*PANAS*)**. The magnitude of the affect was measured on a visual analogue scale ranging from 0 to 10. The scale was anchored by the following descriptors: Most Unpleasant (0); Neutral (5); Most Pleasant (10). All data were plotted as the percentage change from the unstimulated resting or neutral state (PANAS = 5).

In addition to PANAS, subjects were also provided a Visual Analogue Scale for Pain (***VAS***), which had a range from 0 (no pain) to 10 (worst pain). While the VAS was included for the pain-modulation observations (*Experiment 2*, see following text), it was nonetheless administered in *Experiment 1* with the aim of confirming that our affective stimuli themselves were perceived as non-painful by all participants (VAS = 0).

Based on prior experiments [13,14], an inter-stimulus interval of 45 s was used for both stimulus types (vibrotactile and stroking) in order to allow recovery of the skin and avoid adaptation of the neural system. This is consistent with the proposed recovery time for CLTMR function in animals (~30 s), and conforms to the stimulation interval followed in human psychophysical and microneurography studies [11,13,20,21]. To eliminate any effects of auditory and visual cues on subject responses, white noise was delivered through headphones and their vision was obscured.

Experiment 2: Pain modulation

In *Experiment 2*, the effects of stroking and vibration on muscle pain were explored by infusing 5% hypertonic saline (HS; AstraZeneca Pty Ltd, North Ryde, NSW, Australia) into the TA. The HS was administered by inserting a 25 G butterfly cannula through the skin, ~6 cm distal to the tibial tuberosity, which was connected to an infusion pump (model 55–2226, Harvard Apparatus, Holliston, MA, USA). The hypertonic saline was infused (150–200 μl/min) into the TA in order to maintain a clearly perceptible, stable baseline pain for the duration of the infusion (∼ 15–20 min). All subjects were asked to report on the VAS whether stroking (velvet and sandpaper) or vibration (low and high frequency) increased, decreased or had no effect on the overall pain intensity.

Nerve conduction blocks

In compression block experiments, a metal bar was placed distal to the ischial tuberosity in order to apply compression to the sciatic nerve. Somatosensory sensibility was tested within and beyond the innervation territory of sciatic nerve in order to compare and confirm the progression of the block. Myelinated blockade was confirmed by the loss of detection of vibration and cold within the affected region. Vibration sense was tested using the parameters of our test stimuli (20 and 200 Hz, 200 μm). Cold sense was tested by applying a ~15°C brass rod to the skin for 5 s. The preservation of warm sensibility (detection of a ∼40°C brass rod) was taken as confirmation for the intactness of C fibres [22–25]. Additionally, these stimuli were applied to the skin overlying the medial aspect of leg (innerved by femoral nerve), and the contralateral leg, in order to compare sensibilities across the affected and intact areas. Once the block had taken effect, the affective (no experimental pain, *Experiment 1*) and pain-modulatory (during

HS, *Experiment 2*) effects of stroking and vibration were retested in separate experimental sittings.

Statistical analysis

Data are presented as mean ± standard error of the mean (± SEM). In each individual, the responses to stroking and vibration were expressed as an absolute percentage change in affective rating (PANAS, *Experiment 1*) or pain rating (VAS, *Experiment 2*) immediately preceding tactile stimulation. Triplicate trials were treated as independent, sequential events: as triplicate trials of each stimulus combination were statistically indistinguishable, attesting to the reproducibility of the evoked effects, triplicate responses were pooled (within subjects) and averaged across all subjects. Significant changes were detected using 2-way repeated measures analysis of variance (RM-ANOVA) [26]. Where significant differences were indicated ($P < 0.05$), individual groups (control vs. compression) were compared using a Bonferroni correction multiple comparison test. All statistical comparisons were made using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Stroking stimuli

When all fibres were intact, subjects readily distinguished between velvet and sandpaper based on their distinct sensory qualities and never reported them as painful (VAS = 0). However, following compression block, while the subjects could no longer perceive the sensory-discriminative aspects of the tactile stimuli such as onset, motion and pressure–consistent with the blockade of myelinated fibres–they were able to ascribe an affective rating to them. Akin to the intact condition, neither of the stimuli was perceived as painful (VAS = 0) following compression nor were there any visible signs of skin abrasion (e.g. redness) at the stimulation site. In all trials, the affective rating returned to the neutral level (PANAS = 5) before the next trial.

Velvet

In *Experiment 1*, all subjects reported stroking with velvet fabric as being pleasant (Fig 1A) over the range of test stimuli (0.1, 1.0 and 10.0 cm/s). During stroking with each velocity a reproducible sense of pleasantness or positive affect (*0.1 cm/s*: 26.2 ± 5.5%; *1.0 cm/s*: 29.6 ± 4.7%; *10.0 cm/s*: 41.4 ± 4.7%) was reported relative to the absence or neutrality of affect (PANAS = 5 or 100%) whilst the stimulus was in contact with the skin but remained stationary. Following compression, although the subjects were unable to perceive the discriminative aspects of the moving stimulus (i.e. the onset, pressure or textural properties of velvet), they could ascribe pleasantness to velvet-stroking (*0.1 cm/s*: 18.3 ± 5.6%; *1.0 cm/s*: 23.0 ± 6.6%; *10.0 cm/s*: 31.0 ± 7.9%). Importantly, pleasantness ratings were not significantly different between the intact and compression conditions (RM-ANOVA: F = 1.09; $P = 0.32$). While finely graded velocity differences were not observed between the three velocities tested, a significant difference was observed in the pleasantness ratings when comparing the extreme velocities (0.1 cm/s vs. 10.0 cm/s; $P = 0.02$) in the intact condition, but this was not evident following compression ($P > 0.05$).

Following the induction of muscle pain (*Experiment 2*, Fig 1B), stroking with velvet evoked suppression (analgesia) of the overall pain. This effect was stimulus-locked and short-lasting, as the pain intensity returned to levels immediately preceding stroking during the inter-stimulus interval. The *analgesic* effects of velvet-stroking were observed both prior to (*0.1cm/s*: -12.0 ± 5.9%; *1.0cm/s*: -12.3 ± 3.3%; *10.0cm/s*: -16.7 ± 3.3%) and following compression

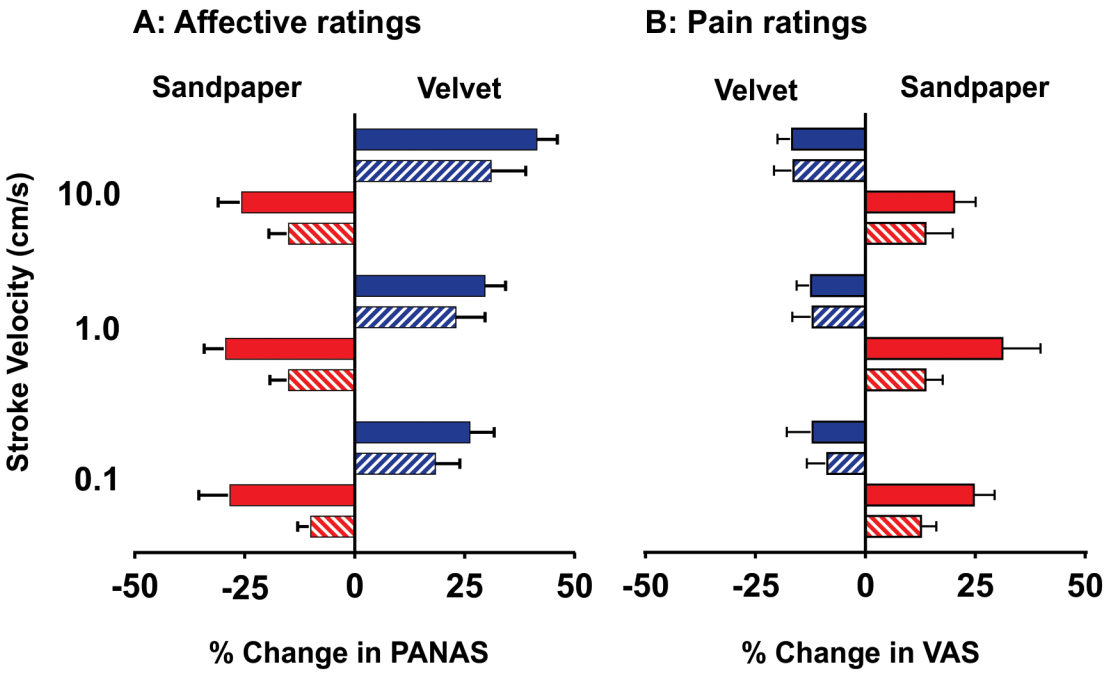

Fig 1. Affective responses (A) to stroking stimuli and their modulatory effects on muscle pain (B) prior to and following compression (mean ± SEM, *n* = 10). A. In response to stroking with velvet (blue bars), subjects reliably reported a pleasant (positive affective) quality, whereas an unpleasant (negative affective) quality was reported in response to stroking with sandpaper (red bars). These opposing effects were observed while all fibres were intact (solid bars) and following compression block of myelinated fibres (hatched bars). **B.** Following the induction of muscle pain, velvet-stroking reduced the overall pain intensity (analgesia), whereas sandpaper-stroking increased the overall pain intensity (allodynia). The analgesic and allodynic effects of velvet- and sandpaper-stroking persisted following the compression block of myelinated fibres (hatched bars).

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blockade (*0.1cm/s*: -9.7 ± 4.6%; *1.0cm/s*: -12.0 ± 4.6%; *10.0cm/s*: -16.3 ± 4.4%). Importantly, the stroking-evoked changes were not significantly different between the intact and compression conditions (RM-ANOVA: F = 0.04; *P* = 0.84). Furthermore, no significant differences emerged as a function of stroking velocity (RM-ANOVA: F = 2.26; *P* = 0.13).

Sandpaper

In *Experiment 1*, all subjects reported stroking with sandpaper as evoking a negative affect (i.e. being unpleasant) but non-painful (Fig 1A). Although stroking with sandpaper was reported as unpleasant over the range of test stimuli, no significant differences were observed as a function of velocity (all fibres intact; *0.1 cm/s*: -28.3 ± 7.1%; *1.0 cm/s*: -29.3 ± 4.9%; *10.0 cm/s*: -25.7 ± 5.4%; RM-ANOVA: F = 0.53; *P* = 0.60). Following compression, although the effect sizes were significantly reduced relative to the intact condition, the subjects retained the capacity to attribute an unpleasant quality to the stimulus (*0.1 cm/s*: -10.0 ± 3.0%; *1.0 cm/s*: -15.0 ± 4.3%; *10.0 cm/s*: -15.0 ± 4.5%; RM-ANOVA: F = 9.69; $P < 0.02$).

Following the induction of muscle pain (*Experiment 2*, Fig 1B), stroking with sandpaper evoked a reproducible increase in the overall pain intensity from a steady pain rating observed prior to stroking across all three stimulation velocities (all fibres intact; *0.1 cm/s*: 24.7 ± 4.8%; *1.0 cm/s*: 31.2 ± 8.7%; *10.0 cm/s*: 20.2 ± 4.9%). No systematic differences were observed as a function of stimulus velocity (RM-ANOVA: F = 0.70; *P* = 0.51). Following compression, all subjects reliably reported increases in the overall pain during stroking with sandpaper (*0.1 cm/s*: 12.7 ± 3.5%; *1.0 cm/s*: 13.7 ± 3.9%; *10.0 cm/s*: 13.7 ± 6.2%; RM-ANOVA: F = 19.24; *P* = 0.002). The only

significant reduction in effect size (44%) between the intact and compression conditions was observed at 1.0 cm/s ($P = 0.0417$).

Focal vibration

When all fibres were intact, all subjects readily distinguished between the two vibrotactile stimuli in the low (20 Hz) and high (200 Hz) frequency range. Subjects were instructed to disregard the vibratory or discriminatory (frequency, intensity and location) aspect of the stimulus and focus on the affective attributes, that is, whether the stimulus evoked a pleasant or unpleasant sensation, or whether it was devoid of any affective quality. All subjects reported low-frequency vibration as pleasant and high-frequency vibration as unpleasant (as rated on PANAS; Fig 2A). Neither of the stimuli was reported as painful (VAS = 0).

A two-way RM-ANOVA revealed that although the magnitude of the effects observed at 20 and 200 Hz was comparable, the sign or direction of the effects was opposing. Furthermore, the amplitude of the effects did not differ between the intact and compression conditions (RM-ANOVA: F = 0.40; $P = 0.54$). Likewise, the opposing effects on muscle pain were statistically indistinguishable between the two conditions (RM-ANOVA: F = 0.0003; $P = 0.99$).

20-Hz vibration

In *Experiment 1*, all subjects perceived the 20-Hz vibration as being pleasant, which was reported as a positive change in the PANAS rating. Fig 2A shows the pooled mean data of triplicate responses for the intact condition (28.6 ± 7.3%) and following compression (22.4 ± 5.1%). In each trial, the affective ranking returned to the neutral level before the next trial.

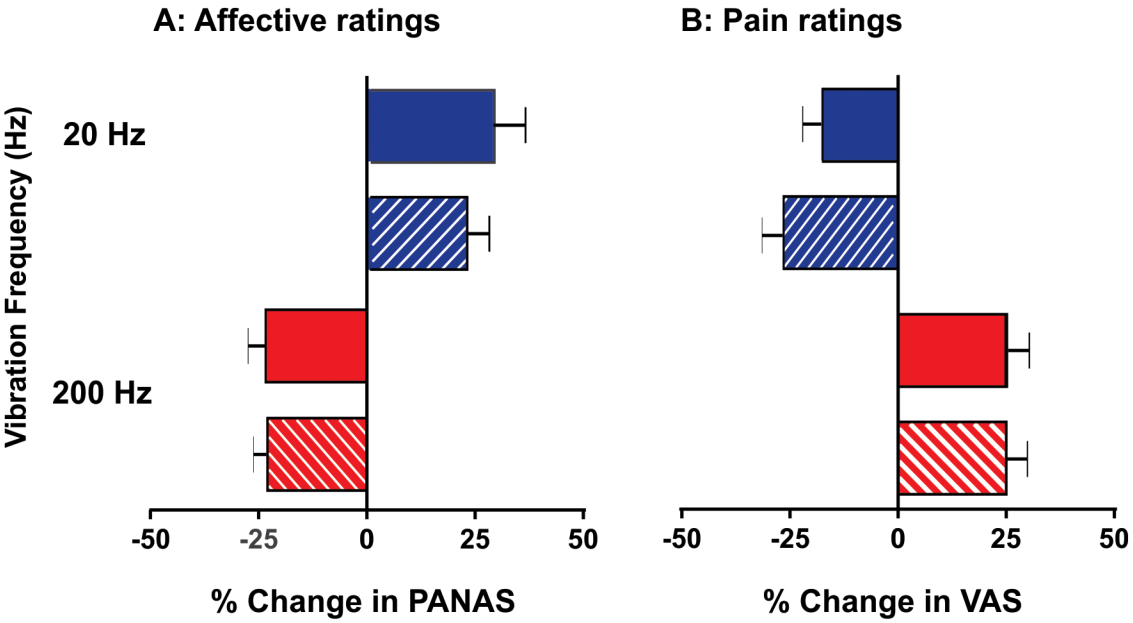

Fig 2. Affective responses (A) to vibrotactile stimuli and their modulatory effects on muscle pain (B) prior to and following compression (mean ± SEM, *n* = 10). A. In response to low-frequency vibration (solid blue bars) subjects reliably attributed a pleasant quality, whereas an unpleasant quality was reported in response to high-frequency vibration (solid red bars). **B.** Following the induction of muscle pain, low-frequency vibration reduced the overall perception of pain (analgesia), whereas high-frequency vibration increased the overall pain (allodynia). The relationship between stimulation frequency, affective regard and pain modulation was preserved following the compression block of myelinated fibres (hatched bars).

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Following the induction of muscle pain (*Experiment 2*, Fig 2B), all individual responses and the pooled mean data showed that the 20-Hz vibration evoked a reproducible reduction in the overall pain both prior to (-17.7 ± 4.2%) and following compression (-26.1 ± 4.7%). In the absence of any superimposed vibration, the HS-induced muscle pain did not vary significantly ($P > 0.05$) throughout the experiment or between the intact and compression conditions. These observations demonstrate that low-threshold C-fibre inputs can elicit analgesia during ongoing muscle pain.

200-Hz vibration

In *Experiment 1*, all subjects reported the 200-Hz vibration as being unpleasant but not painful (Fig 2A). In each case, the overall affective rating was reproduced on at least three occasions both prior to (-22.8 ± 4.0%) and following (-22.4 ± 3.0%) compression block.

Following the induction of muscle pain (*Experiment 2*, Fig 2B), the pooled mean data show that 200-Hz vibration evoked an increase in the overall pain intensity both prior to (24.4 ± 4.9%) and following (24.4 ± 4.8%) compression block. Hence, a stimulus perceived as unpleasant (not painful) manifested as allodynia during ongoing muscle pain. Both negative affect and pain exacerbation persisted while the myelinated fibres were conducting or not, thereby suggesting a role of low-threshold C fibres in mediating these responses.

Discussion

In this study we provide psychophysical evidence that both positive (pleasant) and negative (unpleasant) affective tactile attributes are reliably ascribed to 'everyday' textural surfaces as well as to less familiar stimuli such as sinusoidal vibration. Furthermore, these affective judgments were preserved following the conduction block of myelinated fibres, indicating that affective touch sensations can be sustained by C-fibre inputs alone. Indeed, this was most clearly demonstrated with vibrotactile stimuli where the subjects were unable to detect the discriminative (onset, intensity and frequency) aspects of vibration following the compression block, yet they could reliably ascribe positive and negative affections to 20-Hz and 200-Hz vibration even though the order of stimuli was randomised. Such stimulus fidelity was also observed following the induction of muscle pain: those stimuli perceived as pleasant (velvet and 20-Hz vibration) reduced the overall perception of pain, whereas those perceived as unpleasant (sandpaper and 200-Hz vibration), but not painful, increased the overall pain. The modulatory effects on pain (*i.e.* allodynia and analgesia) persisted when the myelinated fibres were blocked, thereby suggesting that these effects can be mediated by C fibres alone. These results not only confirm our earlier findings that CLTMRs can mediate the allodynic effect of 200-Hz vibration during ongoing muscle pain [13,27], but also provide new evidence that both allodynia and analgesia can be subserved by cutaneous afferents within the C-fibre range. Consistent with our earlier work, we elected to use a two-compartment model, in which pain was induced within the muscle and cutaneous responses tested in the overlying skin, in order to ensure that the observed changes in affective and nociceptive processing were the result of central interaction rather than a change in primary afferent responsiveness within the skin.

In an attempt to disentangle the contribution of different afferent fibre classes for velvet and sandpaper experiments, we selected a range of stimulus velocities including those that have been shown to be optimal for CLTMR activation [11]. As noted in the Introduction, the presence of comparable inverted U-shaped tuning curves for the afferent discharge and the corresponding psychophysical judgments have been previously reported in support of the role of CLTMRs in affective judgments. In our experiments, the use of two textures (sandpaper and velvet), although failing to reveal any finely graded velocity-dependent effects, did reveal

opposing texture-based effects that were reproducible in both intact and compression conditions. How the complex sensory or affective attributes are encoded in the response of an individual or population of C fibres remains unclear. However, the present data demonstrates that both positive and negative attributes can be reliably detected in the presence of unmyelinated fibres with the myelinated fibres blocked.

Even though CLTMRs respond to static touch [9,28], static contact of velvet or sandpaper with the skin was not sufficient to evoke a distinct affect, therefore it appears that the progressive recruitment of multiple units across the skin surface (as with a stroking stimulus) is required in order to generate an affective qualia. In the current study, the use of a controlled mechanical device (RTS) to deliver high-precision (in terms of force, velocity and direction) stimuli to the skin, namely the soft pile of velvet fabric versus the hard grains of sandpaper, resulted in opposing affective qualities. Such considerations are evident in the earlier work where delivering controlled stimuli of varying textures resulted in modulation of the affective rating [29]. One way to explain the opposing affects evoked by moving stimuli is to postulate that each stimulus sets up a differential pattern of afferent discharge based on the spatial distribution of textural elements. In addition, the effects of varying surface textures may well be influenced by skin compliance and frictional forces at the skin-stimulator interface, thus explaining, in part, the lack of a distinct affective quale with static mechanical contact. Studies on compliance encoding have argued for a critical role of large-diameter fibres such as slowly adapting afferents. However, the role of affective coding in this context remains largely unexplored [30,31]. Previous studies [29,32] have conjectured about the involvement of low-threshold C mechanoafferents in affective judgments, but they did not test the contribution of C fibres by using a conduction blockade of myelinated fibres. Our experiments confirmed that subjects could not only detect the affective attributes of touch following the blockade of myelinated fibres, but also reliably discriminate between the opposing affective stimuli.

The demonstration of opposing frequency-dependent affective responses when vibratory stimuli were applied to a fixed point on the skin highlights the complexity of the afferent coding of affect and introduces the possibility of coding strategies based on differential afferent class contributions and/or the pattern of impulse activity initiated at the fixed site of stimulation. In contrast, in the velvet/sandpaper task, judgments appeared to be based on complex spatio-temporal recruitment of afferent activity as the texture was moved across the skin surface. Furthermore, while both tasks (texture and vibration) can generate positive and negative affect, it remains unclear whether the presence of comparable affective responses arises due to complex patterns of spatial-temporal convergence at a spinal or cortical level. However, our observations that the perceived quality of the affect (positive or negative) attributed to a stimulus can reliably predict the modulatory effects on muscle pain (increase or decrease) suggests that both affect and pain may well be processed within closely linked circuits in the central nervous system.

Studies examining the affective processing in a large-fibre deafferented patient revealed a pattern of activation in the insular cortex, and deactivation in the somatosensory, motor, anterior cingulate, parietal association and prefrontal cortices as well as thalamus [10,33]. The deactivation of areas implicated in pain processing has been used to argue for a role of CLTMRs in the suppression of pain. Intriguingly, remarkably similar areas of activation were observed when brushing was delivered to one's own skin as well as others' skin surface, suggesting that 'empathetic touch' or the associated affect can generate comparable patterns of cortical activation [34]. Furthermore, the coupling of multimodal stimuli has shown that high-saliency affective stimuli such as disgusting odours can decrease touch pleasantness [35].

The proposition that CLTMRs normally supress 'pain' inputs needs to be broadened in light of our previous observations where the expression of allodynia–evoked by 200-Hz vibration

and gentle brushing at CLTMR-optimal speeds–in rapid-onset, delayed-onset and chronic pain conditions remained preserved following the preferential blockade of myelinated fibres but was abolished following the preferential blockade of C fibres in the skin [13,14,25,27]. Furthermore, in the current study, we have shown that, following the blockade of myelinated nerves, subjects could reliably detect affective stimuli, which in turn predicted their modulatory effects on muscle pain. Moreover, a unimodal role cannot completely explain the reported correlations between afferent recordings and psychophysical observations that included both positive and negative affective ratings [11]. To resolve this conundrum, further research is warranted into the coding mechanisms of low-threshold C fibres. Consistent with recent molecular/genetic studies where heterogeneity within the CLTMR population has been reported, further investigation into these functionally undefined subtypes is warranted [36,37].

Molecular studies have identified a host of target molecules that, in addition to serving as markers of different fibre classes, may play a critical role in defining the contribution of unmyelinated fibres to synaptic processing and pain modulation [36,38]. For example, CLTMRs project to the inner part of lamina II, a region implicated in the transition from acute to persistent pain and injury-induced mechanical allodynia [39,40]. In addition, CLTMRs co-express the pro-nociceptive glutamate and the analgesic TAFA4 protein [36,41]. The complexity of CLTMR contributions to synaptic processing is further highlighted by the expression of GINIP, a Gα-inhibitory interacting protein, which normally enhances the level of presynaptic inhibition in both TAFA4-expressing CLTMRs and Mas-related G-protein-coupled receptor D-positive (MrgprD+) neurons [38]. In both cases, the use of knock-out models has demonstrated that the loss of either molecule (GINIP/TAFA4) results in pronounced (and prolonged) mechanical hypersensitivity following injury. However, further work is required to determine how the balance between glutamate-mediated excitation, TAFA4 and GINIP-mediated processes can contribute to a shift between positive and negative affect, let alone the opposing modulatory effects observed during muscle pain in this study.

Recent animal work [37] showing that tactile (and cold) allodynia are dependent upon the expression of low-voltage T-type Cav3.2 channels in CLTMRs reinforces our hypothesis that this afferent class contributes to allodynia observed following acute muscle pain, delayed onset muscle soreness and in clinical subjects [13,25]. Beyond the demonstration that selective Cav3.2 knock-out in mice diminished tactile and cold allodynia following injury, the pharmacological blockade of Cav3.2 channels in wild type mice resulted in decreased responsiveness of CTLMRs (increased excitability threshold). Furthermore, in our recent work [42], we have demonstrated that the use of the same calcium channel antagonist abolished experimental cold allodynia in healthy human subjects–providing additional support for the role of CLTMRs in pain processing.

In conclusion, we have provided evidence that low-threshold cutaneous afferent fibres within the C-fibre range contribute to affective and pain processing. Affective tactile sensations, pleasantness and unpleasantness, were evoked using two types of test stimuli with different spatio-temporal properties. Importantly, we found that the affective attributes of tactile stimuli predicted their modulatory effects on pain. That is, the unpleasant stimuli evoked allodynia and the pleasant stimuli evoked analgesia. Following the blockade of myelinated fibres, although the sensory-discriminative aspects of touch were impaired, the capacity to perceive affective touch remained intact. Likewise, following the induction of muscle pain, the capacity of affective stimuli to evoke allodynia and analgesia was preserved regardless of whether the myelinated fibres were conducting or not. Further investigation is warranted into the characterisation of CLTMRs and the coding mechanisms underpinning their dichotomous role in affect-based modulation of pain.

Author Contributions

Conceived and designed the experiments: SS SSN FM DAM. Performed the experiments: SS SSN DAM. Analyzed the data: SS SSN DAM. Contributed reagents/materials/analysis tools: SS SSN FM DAM. Wrote the paper: SS SSN FM DAM.

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