Title

Identification of mechano-sensitive C fibre sensitisation and contribution to nerve injury induced mechanical hyperalgesia

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'what's already known about this topic?'

- C fibres demonstrate increased evoked activity to mechanical stimulation following a traumatic nerve injury.

'what does this study add?'

- Sensitised C fibres have associated alterations in axonal properties to the degree of mechanical hyperexcitability.
- Nerve injury induced enhancement of C fibre evoked activity occurs due to alterations in firing patterns and diminished adaptation rates due to axonal excitation.
- Application of anti-nociceptive galanin to the sensory nerve trunk of naive animals reduces axonal hyperexcitability.

Abstract 250 words

Background: C fibre hyperexcitability is fundamental to chronic pain development in humans and rodents therefore peripheral sensory neuronal sensitisation plays a role in the development of mechanical hyperalgesia. However, the axonal properties and underlying mechanisms that are associated to these chronic pain states still requires investigation.

Methods: Teased fibre electrophysiology of the saphenous nerve was used to identify C fibres in naïve and nerve injured rats. C fibres were identified using electrical stimulation which further provided conduction velocity slowing profiles. Further, in these nerve filaments evoked responses to mechanical stimuli were recorded. Vehicle or galanin were applied directly to the saphenous nerve trunk prior to stimulation.

Results: Increased levels of mechanically evoked activity in mechano-sensitive C fibres was associated to reduced conduction failure, enhanced conduction velocity latency recovery and reduced conduction velocity slowing. Mechanical hyperalgesia developed in nerve injured animals in which mechano-sensitive C fibres demonstrated increased mechanically evoked responses and reduced rate of adaptation. Mechano-sensitive C fibres in nerve injured animals had reduced levels of conduction velocity slowing, enhanced rate of conduction velocity recovery and reduced firing frequency failure versus naïve animals; all hallmarks of enhanced sensory neuronal excitability. Directly applying the anti-nociceptive agent galanin to the saphenous nerve trunk in naive animals led to increased conduction failure, reduced latency recovery rate and increased levels of conduction velocity slowing.

Discussion: Nerve injury induced enhanced neural responses to mechanical stimulation are associated to defined parameters setout by conduction velocity slowing, mediated via axonal processing. Application of galanin inhibits axonal excitability.

Introduction

Neuropathic pain greatly impacts upon the sufferers well-being. Typically described as ongoing pains (burning, pins and needles, pricking pains), in addition to those heightened sensations to evoked pain; allodynia and hyperalgesia. These sensory phenomena arise, in part, due to alterations in the peripheral nervous system either as a result of physical injury, treatment or disease. Primary afferent sensory nerves detect and encode the modality and strength of any applied stimulus (Koltzenburg et al., 1997; Lynn and Carpenter 1982), with the sensory nerve terminal thought to be the fundamental site at which these signals are generated (Carr et al., 2009). Furthermore, the C fibre adapts to the applied stimulus, diminishing the neural response (Andrew and Greenspan 1999; Handwerker et al., 1987) and subsequent reduction in the perceived pain (Schmidt et al., 2000). In rodent models of chronic pain, C fibres elicit greater levels of neural activity (ongoing and/or evoked) (Chen and Levine 2001). This elevated level of action potential traffic is received by the central nervous system (CNS) resulting in central sensitisation and increased–pain (Li et al., 1999). Specialised transduction mechanisms are responsible for activation thresholds and/or evoked activity of C fibres (Ahlgren et al., 1997; Chen and Levine 2003). Diminished regulation of action potential firing frequency rates in neuropathic pain (Chen and Levine 2003; Djouhri L 2001; Gemes et al., 2013; Serra et al., 1999; Taguchi et al., 2010) have recently been documented to be a plausible explanation for such a phenomena. However, processes that regulate axonal conduction allowing for the faithful trafficking of action potentials also play a large role in mechanical hypersensitivity (Gemes et al., 2013; Sun et al., 2012). C fibre nociceptor mechanical sensitisation through intradermal application of nerve growth factor is accompanied by axonal excitability (Hirth et al., 2013; Obreja et al., 2011b). Therefore alterations in the temporal firing patterns of C fibres, particularly in reference to mechanical stimulation, and axonal conduction parameters need to be considered in the generation of mechanical hyperalgesia.

Here in this study the relationship between axonal characteristics are investigated to outline defined parameters of peripheral sensory neuronal sensitisation and mechanical hyperalgesia. The neuropeptide galanin is upregulated post-nerve injury in the peripheral sensory nervous system and has antinociceptive actions by blunting mechanical hyperalgesia via inhibition of sensory neuronal excitability (Holmes et al., 2003; Hulse et al., 2011). To date its direct impact upon axonal properties has not been investigated. Manipulation of axonal function was carried out via the application of galanin to the sensory nerve trunk to demonstrate C fibre excitability can be attenuated through regulation of axonal processing. The hypothesis is that in a model of mechanical hyperalgesia C fibre sensitisation is

associated with axonal markers of sensitisation, further defining axonal excitation; and that this can be inhibited via galanin application.

Methods

Animals

Forty seven male Wistar rats (250-350g) underwent procedures regulated and authorised by the UK Home Office and the Animal (scientific procedure) Act 1986. Animals were housed in 12:12hr light dark cycles, with access to food and water ab libitum.

Nerve injury surgery

Partial saphenous nerve ligation injury (PSNI) surgery was carried out on fifteen wistar rats under isoflurane (in O_2) anaesthesia (Hulse et al., 2010). Using a sterile silk suture (size 4.0) ~50% of the saphenous nerve was tightly ligated. The wound was closed and the animal well-being was monitored on recovery. Animals were left three days post-surgery prior to behaviour or electrophysiogical experimentation.

Nociceptive Withdrawal Behaviour

Rodents were habituated to the testing environment and experimental procedures prior to behavioural testing. Von Frey hairs were applied a total of five times to each hindpaw with a range of forces applied to elicit 0% to 100% withdrawals.

Primary Afferent Electrophysiology

Setup was carried out as previously described (Dunham et al., 2008; Hulse et al., 2011; Hulse et al., 2014). Anaesthesia was induced via intraperitoneal injection (60mg/kg sodium pentobarbital). For anaesthesia maintenance, intravenous (i.v) cannulation of the jugular vein was carried out allowing for i.v. anaesthesia administration (20mg/kg/hr sodium pentobarbital). An incision was made along the inguinal fossa region to expose the saphenous nerve on the right hindleg. Skin was attached to a ring to form a bath and filled with mineral oil. The saphenous nerve was placed on a dental mirror. Using fine forceps/scalpel blades fine nerve filaments were dissected away from the main nerve trunk. These were placed on bipolar platinum recording electrodes. Using in house amplification and filtering equipment recordings were visualised and acquired digitally via a micro1401 (Cambridge Electronic Design, UK) on a computer through the use of Spike v7 (Cambridge Electronic Design, UK).

Mechanically sensitive units were identified initially through brush and/or pinch searching of the medial surface of hindpaw. Individual primary sensory afferents (same process for naïve, nerve injured and galanin treated experiments), with non-overlapping receptive fields, were confirmed via electrical stimulation (conduction latency and identification dependent on spike shape). In the instance of PSNI animals, those units with receptive fields were deemed ‘intact’ injured sensory nerve fibres as previously characterised (Djouhri et al., 2012; Hulse et al.,

2010). Multiple units were recorded per animal to minimise animal usage number where possible as outlined by the Home Office and the Animal (scientific procedure) Act 1986.

Electrical stimulation (0.5ms duration, at max. 100V intensity, rate 0.3Hz) was directly applied to the receptive field via stimulating needle electrodes. Conduction velocity for identified afferents was defined as C-fibres <2m/s (Dunham et al., 2008). All units with conduction velocities greater than 2m/s were classified as A fibres. A fibres were not included in this investigation. Conduction velocity was determined via acquisition of latency and the distance between electrode sites (receptive field and recording electrodes). Electrical stimulation threshold was attained and upon confirmation 1.5x threshold was used subsequently for high frequency stimulation (conduction velocity slowing (CVS) (Obreja et al., 2011b)).

The CVS stimulation protocol (Serra et al., 2011) was as follows; 180s 0.25Hz, 180s rest, 360s 0.25Hz, 180s 2Hz, 420s 0.25Hz. The outlined electrical protocol allows for the acquisition of those parameters as setout in Fig.S1 (raw data extracted from the recording trace). Fig. S1 highlights a single identified C fibre (overlay of evoked action potentials top panel) responding to peripheral (skin) electrical stimulation; initially 0.25Hz then 2Hz as indicated by the arrow. This C fibre demonstrates conduction velocity slowing (CVS) overtime (x-axis=time (s) in all panels; Fig. S1 Bottom panel), Diagrammatic explanations of the CVS profile identifies CVS, % conduction failure and period of recovery. As well as conduction velocity slowing (peak CVS at 2Hz stimulation) (Serra et al., 1999), conduction latency recovery rate (15^{th} electrical stimulation following the switch to 0.25Hz following high frequency 2Hz (Serra et al., 1999)) and conduction failure (percentage of successful action potential generated (maximum total 360) over 180s 2Hz period) (Hirth et al., 2013)) were determined.

Following conduction velocity identification 2 minutes of baseline/ongoing activity was recorded with no further stimulus applied. Subsequently, receptive field was stimulated with pinch, the movement of a brush across the surface and 3 applications of each applied von Frey hair (Linton instruments, UK) (Chen and Levine 2003; Lynn and Carpenter 1982). Von Frey hairs are a well-established methodology for nociceptive behaviour and electrophysiological experimentation (Chen and Levine 2003; Hulse et al., 2010; Hulse et al., 2011; Lynn and Carpenter 1982) providing a robust, reliable stimulation protocol and additionally provides dexterity to application across the hindpaw. To determine mechanical activation thresholds for mechanically sensitive C fibres an up and down method was used to determine mechanical activation threshold. Mechanical thresholds were determined as the lowest von Frey hair applied that elicited a robust (>3 action potentials) reproducible response (Dina OA 2004; Dunham et al., 2008). Nociceptive (high threshold mechano-sensitive (HTM))

afferents responded to pinch when applied to the receptive field but not brush. Rigorous definition of low threshold (LTM) mechano-sensitive afferents were determined as those C fibres that were responsive to brush (Hulse et al., 2010; Shea and Perl 1985). For evoked responses a standard set of 2, 4, 10, 26 and 60g von Frey hairs were applied three times, with neuronal discharge per stimulus determined as mean firing rate (Hz) of the 5 second stimulation. Degree of adaptation was represented by timelocked evoked activity (Hz) to 1s bins for the first 3s of 10g stimulus application. All identified units with activation thresholds above the 180g mechanical stimulus/non-responsive to those applied stimuli (i.e. silent nociceptors) were not included in this analysis.

For drug/vehicle control studies; one further electrical stimulation protocol for CVS was undertaken. Test solution (ringers or Galanin (10µM as previously described (Hulse et al., 2011)) was applied directly to the exposed nerve trunk towards the recording site. Galanin was made up in physiological saline.

Data Analysis and Statistics

All data is represented as mean±SEM, unless otherwise stated with these described in supplementary table 1. Microsoft Excel 2010 and Graphpad Prism 5 was used for all offline analysis and figure construction. Mechanically evoked activity was determined by deducting ongoing activity from the total activity recorded during the mechanically stimulated phase. From these the three applications of each vF hair a mean value was determined, which is represented in the text and figures. Nociceptive behavioural testing and mechanically evoked activity was analysed with One-way ANOVA with post Bonferroni test and/or trend for linearity, Two-way ANOVA with post Bonferroni test. From conduction velocity slowing curves; the peak slowing value was calculated. Conduction latency from the final electrical stimulation from the 2Hz phase over the distance (mm) between recording and stimulating site was then recorded and conduction velocity calculated. The %CVS value was determined from baseline CV vs peak slowing CV (latency change from baseline 0.25Hz stimulation/360th 2Hz stimulation*100). For the recovery to baseline latency value; the 15th stimulation during 0.25Hz following the 2Hz period was taken and determined as a percentage of the baseline conduction velocity. Following frequency failure were calculated as the total number of action potentials elicited between time 0s of the onset of the 2Hz (high frequency stimulation period) over maximum number of stimulations during 2Hz phase (360). All slowing, recovery and conduction failure were calculated and used as appropriate Mann Whitney or Paired T test. Spearman coefficient and nonlinear regression curves were performed for comparison between appropriate groups taken from the following: CV, mechanical activation threshold, mechanical evoked activity, conduction velocity recovery and conduction failure. To subclass

those C fibres identified in Naïve animals a cut off value was determined from two standard deviations away from this PSNI mean %CVS (18.53%CVS ± 2x 5.03=cut off value 28.59%CVS). Any unit that had a higher CVS than this were determined as high %CVS and anything within that a low %CVS. Those animals used for behavioural analysis were used for electrophysiological experimentation.

Results

Mechanical characterisation and electrical profiling of the mechano-sensitive C fibres in naïve animals

From naïve animals a total of 25 mechanically sensitive C fibres (0.55±0.034m/s) were isolated from the saphenous nerve, all with receptive fields in the hindpaw below the ankle joint. Initial characterisations here of the mechano-sensitive C fibre nociceptors were to determine whether the level of CVS was related to the extent of mechanical sensitivity. C fibres were split into those classifications that were LTM (brush sensitive) (n=10; 40%) or HTM (not brush sensitive) (n=15; 60%). The degree of CVS (Fig. 1A) did not relate to those mechanical characterisations as outlined. Furthermore, CV (Fig. 1B) did not differ between LTM and HTM groups.

Relationship between mechanically evoked activity and slowing profile attributes

The degree of %CVS from the C fibre population was associated to the mean neuronal activity evoked from an application of a 4g mechanical stimulus to the receptive field ($r=-0.57$; $p<0.01$, Spearman, Fig. 1C). Thus mechano-sensitive C fibre afferents with less %CVS had greater mechanically evoked responses to a 4g stimulus. In addition, those C fibres with less following frequency failure (high percentage of successful action potentials) demonstrated a greater level of evoked neuronal response to 4g ($r=-0.46$, $p<0.05$, Spearman, Fig. 1D), with a trend at 10g ($r=-0.43$, $p=0.06$, Spearman, Fig. 1E). Furthermore, those with less %CVS have a greater recovery rate ($r=-0.46$, $p<0.01$, Pearson, Fig. 1F). This demonstrates that subsets of mechano-sensitive C fibres have differing excitability profiles, with those 'primed' C fibres (i.e. high degrees of mechanically evoked activity) demonstrating higher ability to produce high frequency action potential bursts in response to electrical and mechanical stimulation. It has been documented that silent nociceptors have the highest degree of %CVS, in addition high degrees of following frequency failure at 2Hz (Hirth et al., 2013). However, those units fail to respond to mechanical stimulation and in this study all C fibres had an activation threshold of less than 30g and/or responsive to brush, and as such all units presented in this study have been deemed mechano-sensitive.

Mechanical hyperalgesia in a rodent model of neuropathic pain

The PSNI nerve injury model~~(Hulse et al., 2008; 2010)~~(Hulse et al., 2008; 2010) (3 days post nerve injury) leads to mechanical hyperalgesia, whereby significantly greater number of nociceptive withdrawals in the PSNI injured animals in response to those noxious mechanical

stimuli applied (15g and 26g) compared to those response values of naïve uninjured age, gender matched controls (Fig. 2A; *** Two way ANOVA with post Bonferroni).

Enhanced mechanically induced C fibre nociceptor discharge in a rodent model of neuropathic pain

In a rodent model of neuropathic pain mechanically evoked neuronal activity from mechano-sensitive C fibres was enhanced 3 days following nerve injury (4g and 10g vF hairs; *p<0.05, ***p<0.01 two way ANOVA with post Bonferroni test) compared to those mechanically induced neuronal responses taken from C fibres in naïve animals (Fig. 2B, example PSNI C fibre Fig. S2B). Those C fibres identified in the nerve injured animals demonstrated a steep increase in evoked responses to noxious mechanical forces, reaching plateau (preferred model fit = sigmoidal curve fit vs linear, p<0.001) in complete contrast to the linear responses identified in the naive animal (#p<0.01 10g vs 26g and 26g vs 60g;***p<0.001 One way with linear trend post test) (Andrew and Greenspan 1999). Conduction velocity was significantly reduced in the PSNI group versus naïve animals (*p<0.05, Fig. 2C). Furthermore, in naïve animals C fibres responseded to mechanical stimulation (10g) and subsequently. neural response declined during the applied mechanical pressure; termed adaptation (Fig. 2C; S2A). However, in the nerve injured group the mean evoked activity was greater over the duration of the applied stimulus when compared to naive animals; thus the rate of adaption (decline of evoked response) was less in the PSNI group (Fig. S2B). The PSNI group had enhanced firing frequency at timebin 1-2s during the period of mechanical stimulation versus naive C fibres (**p<0.01, Fig. 2D).

Enhanced mechanical evoked activity in C fibre afferents following nerve injury

To note, those HTM and LTM C fibres identified here in the naive animals and those C fibres in the PSNI group (n=7) did not demonstrate any signs of ongoing activity (cut off 0.1Hz (Shim et al., 2005)). The total extent (area under curve) of the CVS profiles identified for mechano-sensitive C fibres in PSNI animals was less than that found in naive animals (*p<0.05; Fig. 2E). As defined in Figure 1, C fibres identified in naïve animals with less %CVS were deemed more excitable and were comparable to the PSNI population in relation to mean mechanically evoked neural discharge. Those C fibres in naïve animals with high %CVS had significantly less mechanical neuronal evoked activity discharge (p<0.01, Fig. 2F).

Mechano-sensitive C fibre afferents in nerve injured animals (Fig. 3A&B) had reduced levels of %CVS compared to those LTM (Fig. 3A) and HTM (Fig. 3B) mechano-sensitive C fibres in naïve animals. LTM (Fig. 3C) and HTM (Fig. 3D) mechano-sensitive C fibres in naïve animals had a high degree of following frequency failure compared to those mechano-sensitive C fibres

in the PSNI group (Fig. 3C&D). Further, the latency of recovery back towards the baseline conduction velocity following 2Hz electrical stimulation was shorter in those mechano-sensitive C fibres identified in PSNI animals (Fig. 3E&F) when compared to those LTM (Fig. 3E) and HTM (Fig. 3F) mechano-sensitive C fibres in the naïve group.

Galanin application altered the conduction velocity slowing profile

Galanin was applied to the peripheral nerve at a site situated between stimulation and recording sites in naïve animals. This led to an inhibition of C fibre neuronal responses during high frequency electrical stimulation (CVS protocol). The degree of CVS was increased following galanin application (Fig. 4A) and greater following frequency failure per 2Hz electrical stimulation protocol (Fig. 4B) compared to before drug values in naive animals. The rate of conduction recovery towards baseline values following periods of 2Hz high frequency electrical stimulation was also inhibited (Fig. 4C) in naive animals. Vehicle controls were undertaken in naive animals and led to no change in the CVS profile in a control group of mechano-sensitive C fibre nociceptors (Fig. 4D).

Discussion 1500

Data here supports the notion that mechano-sensitive C fibres are sensitised following nerve injury. Enhanced evoked activity and reduced levels of adaptation of C fibres in response to noxious mechanical stimulation can be regulated by axonal hyperexcitability, in this case via galanin application. Misregulation of such processing contributes to neuropathic pain development; particularly in reference to mechanical hyperalgesia.

Mechano-sensitive C fibre nociceptor firing properties in response to mechanical stimulation

Primary afferent C fibre nociceptors allow the detection and transmission of information regarding tissue damaging stimuli to the central nervous system. Mechano-sensitive C fibres respond and discriminate high intensity stimulation through graded bursts of action potentials and subsequent adaptation of response. Therefore the sensory nerve has the ability to modulate this high frequency neural activity through manipulation of firing patterns (Gemes et al., 2013) by limiting the interspike interval (duration between elicited action potentials) (Chen and Levine 2001; Sun et al., 2012). Data here demonstrates that differing mechano-sensitive C fibres have varying degrees of firing properties. Suppression of axonal excitability i.e. reduced rate of latency recovery and greater following frequency failure rates lead to reduced mechanical evoked activity. However, as demonstrated there are primed subsets that have enhanced ability to recover during noxious or high frequency stimulation delivering high degrees of neural mechanically evoked responses. The summation of this C fibre grouping can be excited by high frequency electrical stimulation trains applied to the skin, with those C fibres recorded in animals and humans (Hirth et al., 2013; Obreja et al., 2011b). This enhances perception of pain (Obreja et al., 2011a), and with the addition of nerve growth factor there is a further enhancement of the pain score (Obreja et al., 2011a).

CVS is utilised to identify C fibres through conduction latencies and allows delineation between differing afferent subtypes dependent upon the maximal degree of CVS (Serra et al., 1999). These studies provide further clarification of those neuronal markers/classifications of sensory neuronal sensitisation versus CVS and C fibre mechanical hypersensitivity. Here CVS profiles between mechano-sensitive subgroups (LTM and HTM units) were comparable. However, these findings are in stark contrast to those studies investigating human and animal CVS profiling of C fibre nociceptors that have identified discrete mechano-populations (Hirth et al., 2013; Obreja et al., 2010; Taguchi et al., 2010). These discrepancies could be due to differing populations of neurons surveyed in this study versus those previously published (Gee et al., 1996; George et al., 2007; Taguchi et al., 2010), with data here extensively dependent upon mechanical stimulation and principally brush which has not been intensely studied in previous studies. However, all CVS profiles recorded are within the documented ranges in all CVS

studies (Gee et al., 1996). Therefore these parameters outlined need to be considered for those research and clinical environments to enable the understanding of such sensory nerve phenomena in relation to identification of sensory neuron subtype and sensitisation investigation.

Sensitisation of mechano-sensitive C fibre nociceptors in mechanical hyperalgesia

This study investigates extensively those neural characteristics as explored with CVS in relation to mechanical stimulation and applies them to a neuropathic pain setting. C fibre nociceptors undergo alterations to neuronal excitability; evoked and/or ongoing activity in neuropathic pain models (Chen and Levine 2001; Chen and Levine 2003; Hulse et al., 2010). However, despite C fibre nociceptor sensitisation, activation thresholds are in a large proportion of cases unaltered in rodent models of neuropathic pain (Chen and Levine 2001; Chen and Levine 2003; Hulse et al., 2010) highlighting that the degree of action potentials generated and conveyed (evoked/ongoing activity) by these sensory neurons is integral to chronic pain development. Temporal alterations in firing patterns have been documented particularly with fluctuations in inter-spike intervals allowing for increased firing frequency in chronic pain models (Chen and Levine 2003; Gemes et al., 2013). This leads to reduced adaptation and thus enhanced mechanically induced evoked activity identified in C fibres in neuropathic pain models. Here this is supported with markers of axonal hyperexcitability and the ability for the sensory neuron to allow faster firing rates, accompanied by high mean mechanically evoked firing rates. This suggests changes in the intrinsic activity of axonal conduction processes. Though these conduction properties may regulate ongoing activity, in this study no C fibres with ongoing activity were observed therefore those links cannot be commented upon in this instance. To note the population of C fibres is heterogeneous, encompassing high and low threshold mechano-sensitive subtypes as documented here, as well as those deemed as silent nociceptors, which are not responsive in an uninjured naïve subject. Silent C fibres (CMI) (Hirth et al., 2013; Kleggetveit et al., 2012) as well as nociceptive mechano-sensitive C fibres (Djouhri et al., 2012) are sensitised (ongoing activity/greater evoked activity) in pathology therefore are a significant contributing factor in chronic pain. Recent publications strongly support that silent nociceptors are the prime candidates for this sensitised C fibre subset in neuropathic situations (Hirth et al., 2013; Kleggetveit et al., 2012) with decreased %CVS and associated ongoing activity present in human neuropathic pain patients (Kleggetveit et al., 2012). However, in nerve injured groupings it is difficult to ascertain the origin of classification for a particular fibre type, and further, whether it has developed sensitisation characteristics. Therefore in many instances classifications are based upon using those same parameters for both naive and neuropathy groups (Djouhri et al., 2012; Hulse et al., 2010) as done so in this study.

Current dogma perceives that peripheral sensitisation, demonstrated through elevations in evoked activity (Chen and Levine 2003) and/or ectopic firing (Shim et al., 2005), is the neuronal correlate of chronic pain. Here the elevated ability to allow increased firing frequency and the successful propagation of increased action potential trains is integral for this information to be received by the CNS and subsequent neuronal potentiation that occurs in chronic pain. Therefore those neural attributes that regulate neuronal firing patterns through reduction in firing rate, are seemingly 'switched off' in neuropathic pain. Differing sites of the peripheral sensory nervous system have fundamental roles in modulating afferent input into the CNS, with these including the regulation of action potential initiation, differing degrees of adaptation and consequent propagation (Hirth et al., 2013). The phenomenon of neuronal excitation (following frequency failure/CVS/shortened period to recovery) has a number of possible causes with varying neuronal structures/locations i.e. free nerve endings and axonal branch points, calculable to the initiation and processing of high frequency sensory inputs (Carr et al., 2009; Gemes et al., 2013). Action potential failure is largely dependent upon distance/or discrete axonal localisation, with increased conduction distance increasing the chance of action potential failure (Sun et al., 2012; Zhu et al., 2009). Prolonged/extended periods of potassium channel dependent hyperpolarisation (Gemes et al., 2013; Sun et al., 2012) increases conduction failure, with this potassium channel dependent failure greatly reduced in models of neuropathic pain (Gemes et al., 2013). However, few studies have categorically altered sensory neuronal output via exclusively targeting axonal structures, particularly *invivo* (Sun et al., 2012). Therefore to deduce the role of such actions the neuropeptide galanin was targeted. Galanin is upregulated in the peripheral nervous system following nerve injury in both the neuronal soma and axonal structures at the site of injury (Armstrong et al., 2008; Hulse et al., 2008). Galanin is also of interest due to the ability to inhibit nociceptor activity (Hulse et al., 2011) plausibly through activation of potassium channels (Parsons et al., 1998), with high levels of galanin (Holmes et al., 2003) (Hulse et al., 2011) preventing neuropathic pain. Galanin inhibited axonal excitability through application directly to the nerve trunk placed between the stimulating and recording site. Action potential conduction failure is largely dependent on changes in axonal membrane potential (Sun et al., 2012), though these actions of galanin can only be speculated, the involvement of potassium channels cannot be ruled out. Therefore modulating intrinsic neuronal excitability through targeting axonal processes provides further understanding of how the C fibres regulate nociceptive stimulation and leading to alternative mechanisms to develop and apply novel analgesics.

Data here supports the hypothesis that changes in the ability of the sensory nociceptor to regulate high frequency trains of action potentials, is heavily involved with mechanical

hyperalgesia. However, following nerve injury these systems that regulate axonal excitability are altered allowing for high frequency firing rates thus engaging peripheral sensory neuronal sensitisation and resulting mechanical hyperalgesia. This process is in part regulated by galanin.

Author Contributions

RH designed and undertook behavioural and electrophysiological experiments. RH wrote the manuscript.

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Figure Legends

Figure 1 –

The extent of mechanical evoked activity by mechanically sensitive C fibres is associated to the degree of conduction failure and conduction slowing. Non-brush sensitive (n=15) and brush sensitive (n=10) c fibres demonstrated no differences in [A] conduction velocity slowing and [B] conduction velocity. [C] Those C fibre that had greater levels of mechanically evoked activity (4g) had less conduction velocity slowing (p=0.01 Spearman coefficient, n=25). [D] Increased following frequency firing was associated with increased mechanical evoked activity to 4g ($p<0.05$, Spearman coefficient, n=25) and [E] 10g (p=0.06, Spearman coefficient, n=25). [F] Additionally, those mechano-sensitive units that had greater levels of conduction velocity slowing also had reduced capacity for conduction latency recovery (p=0.0046, Pearson coefficient, n=25).Broken line represents non-linear regression.

Figure 2 -

PSNI induced mechanical hyperalgesia and alterations in C fibre firing patterns in nerve injured animals.

[A] Three days post partial saphenous nerve ligation injury there are increased numbers of nociceptive withdrawals to a mechanical stimulus in PSNI injured rats compared to naïve controls (n=5 per group, *** $p<0.001$ Two way ANOVA with post Bonferroni test; naive animal comparison #$p<0.01$ 10g vs 26g and 26g vs 60g;***$p<0.001$ One way with linear trend post test). [B] Mechanically sensitive C fibres afferents in naïve animals encoded mechanical stimuli with increasing mechanically induced evoked activity ($r=0.92$, $p<0.05$ Pearson; curve not shown). Mechanically sensitive C fibres afferents identified in the PSNI group had elevated levels of mean mechanically evoked activity to 4g and 10g vF hairs compared to those investigated in naïve rats (*$p<0.05$, ***$p<0.001$ Two way ANOVA with post Bonferroni test). [C] C fibres following PSNI surgery had reduced conduction velocity versus those in the naïve animal (*$p<0.05$, Unpaired T Test). [D] Furthermore, these C fibres from the PSNI group demonstrated significantly greater mean mechanically evoked activity over the duration of stimulus application (time bin 1-2s) versus the naive group. Data represents data from 10g vF hair (*$p>0.05$ Two way ANOVA; open squares naive animals and filled squares PSNI animals). [E] Summary of those CVS curves in both groups indicating significantly greater CVS in naive mechano-sensitive C fibres compared to those in the PSNI group ($p<0.05$, unpaired T test, under curve values, total afferent number n=25). [F] Those excitable C fibres with less %CVS had comparable mean mechanically evoked discharges to 4g to the PSNI group, however those deemed less excitable due to higher %CVS had significantly less evoked activity versus the PSNI group (**$p<0.01$, One way ANOVA with post Bonferroni test).

Figure 3 –

Comparison of mechano-sensitive C fibres in naïve and PSNI injured rats. [A] PSNI rats had a reduced level of conduction velocity slowing compared to those LTM (*p<0.05, Mann Whitney test, n=7) and [B] HTM (**p<0.01, Mann Whitney test) C fibres in naïve control rats. A greater number of successful action potentials were recorded per high frequency stimulation protocol in PSNI versus [C] LTM (p=0.06) and [D] HTM (*p<0.05, Mann Whitney test) C fibres in naïve rats. [E] C fibres in PSNI rodents had an increased rate of recovery back to baseline conduction velocity following high frequency stimulation versus LTM (**p<0.01, Mann Whitney test) and [F] HTM (*p<0.05, Mann Whitney test) C fibres in naïve rats.

Figure 4 –

Galanin modulates C fibre electrical conduction velocity profiling. Application of galanin to the saphenous nerve trunk in naive animals led to [A] increased conduction velocity slowing (**p<0.01, Paired T test, n=6) and [B] increased conduction failure (*p<0.05, Paired T test). [C] Galanin also led to reduced level of conduction latency recovery (*p<0.05, Paired T test). [D] Saline control did not alter the slowing profiles of C fibres during high frequency electrical stimulation (n=3).