LOW-THRESHOLD UNMYELINATED MECHANORECEPTORS: A NOVEL SUBSTRATE OF ALLODYNIA IN HUMAN SKIN

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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.

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ABSTRACT
The human hairy skin is innervated by a very ancient system of slow-conducting low-threshold mechanoreceptors termed C-tactile fibres (CTs). Intriguingly, even the existence of CTs in the distal limbs (non-hairy/glabrous skin) remains to be established, let alone their functional significance in the neural system. In this thesis, we examined the function of CTs through the prism of pain and its multifarious expressions, in particular touch-evoked pain (allodynia). In Paper I, we showed that the deep somatic pain generated by infusion of hypertonic saline (HS: 5%) into the tibialis anterior muscle is enhanced by concurrent application of vibration (200 Hz-200 µm) and brushing (1.0 and 3.0 cm s⁻¹) to the overlying hairy skin. In Paper II, we demonstrated that a comparable expression of allodynia elicits when vibration and brushing are applied across skin types and spinal segments – glabrous skin of fingers and hairy skin of dorsal forearm – during HS-infusion into the flexor carpi ulnaris muscle. In Paper III, we showed that vibration evokes pain/allodynia following eccentric exercise-induced muscle soreness (no resting pain). Furthermore, a cognate expression of touch-evoked allodynia was observed in a clinical subject with activity-triggered heel-pain without exposure to eccentric exercise. In Paper IV, we demonstrated that, in the presence of HS-induced cutaneous pain, the application of vibration to an adjacent region of skin generates allodynia. In all four papers, the vibration- and brush-evoked allodynia persisted following conduction block of myelinated afferents by compression. In contrast, the effect was abolished during conduction block of cutaneous C fibres by injecting a local anaesthetic into the skin stimulation by vibration. Collectively these psychophysical observations provide the first human evidence that CTs arising from hairy skin, and their functional counterparts in glabrous skin, contribute to mechanical allodynia. This phenomenon appears to be reproducible in perceptible (HS-induced), imperceptible (exercise-induced) and pathological pain-states.

Keywords: C fibre; pain; allodynia; hypertonic saline; eccentric exercise; vibration
THESIS STRUCTURE

The work presented in this thesis provides a review of low-threshold cutaneous mechanoreceptors with special emphasis on C mechanoreceptors. In addition, detailed psychophysical observations of vibration- and brush-evoked pain (allodynia) in general, and the peripheral origin of this effect in particular, are provided as a series of four papers (see below). These papers are either published (Paper I), amended based on reviewers’ comments (Papers II & III) or in process of submission (Paper IV), and adverted to in the text by their Roman numerals.


II. **Nagi SS** & Mahns DA. Mechanical allodynia in glabrous skin mediated by low-threshold cutaneous mechanoreceptors with unmyelinated fibres. *Experimental Brain Research* *

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IV. **Nagi SS** & Mahns DA. Do C-tactile fibres have a role in generation of mechanical allodynia during cutaneous pain? *Manuscript*.

*In accordance with reviewers’ critiques, Papers II and III have been revised. We are in the process of submitting the modified versions (presented as part of this thesis in the prescribed formats) to the respective journals for further consideration.*
CONFERENCE ABSTRACTS

Oral presentations


Poster presentations


TABLE OF CONTENTS

INTRODUCTION ........................................................................................................... 1

1. Review of classical mechanoreceptors .............................................................. 2
   1.1 Slow-adapting receptors ................................................................................ 3
   1.2 Fast-adapting receptors ................................................................................. 5

2. Peripheral origins of vibrotactile sensibility ..................................................... 8

3. Low-threshold unmyelinated fibres ................................................................. 9
   3.1 Receptor characteristics ............................................................................. 11
       Responses to mechanical stimuli ................................................................. 12
       Resistance to use-dependent inactivation .................................................... 15
       Responses in a patient with sensory neuronopathy syndrome ................... 16
       Spurious thermoreceptors ........................................................................... 17
   3.2 Chemical phenotype ..................................................................................... 18
   3.3 Spinal projections ......................................................................................... 22
   3.4 Cortical projections ...................................................................................... 24
   3.5 Functional significance ................................................................................. 25
       A critique of pleasant-touch hypothesis ...................................................... 27
       CT-functionality in ‘pain’ context ................................................................. 28

4. CT-mediated allodynia: a précis of findings across four papers ................. 29

Acknowledgements .................................................................................................. 32

References .................................................................................................................. 33
INTRODUCTION

It is widely appreciated that the cutaneous neurocircuitry consists of a myriad of receptors that perform a range of functions, from banal to transcendent. Typically, the classification of these receptors is based on four cutaneous modalities, namely touch, pain, temperature and itch. Touch, in particular the sensory-discriminative facet, subserves the perception of pressure, vibration/texture, stretch and, in case of hairy skin, displacement of hair follicles. Large myelinated mechanoreceptors are well recognised as the underlying neural substrate of such tactile expressions. Conversely, little is known about the peripheral origin of the affective/emotional dimension of touch. In this thesis, the focus is on a rather enigmatic population of mechanosensitive afferents termed C-tactile fibres (CTs) in humans, and C low-threshold mechanoreceptors (CLTMRs) in other species. This afferent type was discovered about seventy years ago in the skin of cat. Over the years, the CLTMRs have generated little interest with doubts over their existence in humans, questions about their functional significance (in the tactile domain) in presence of their myelinated counterparts, and technical difficulties in identifying and recording from them, to name a few deterrents. However, some twenty years ago, it was demonstrated that CTs innervate the human hairy skin, yet the question of their functional relevance has remained unresolved. A number of hypotheses have been put forth over the years, including their role in tickle, itch and, more recently, pleasantness/pleasure associated with skin-to-skin contact between individuals. The pleasant-touch hypothesis has drawn considerable interest for its seemingly intuitive role or pertinence to physical and social well-being, and the interoceptive system. Furthermore, on the basis of this hypothesis, it was conjectured that activation of CT fibres can elicit pain relief. Conversely, a recent study in mice demonstrated touch
hypersensitivity mediated by CLTMRs – a phenomenon termed *mechanical allodynia* – in inflammatory and neuropathic ‘pain’ conditions. We observed, more or less contemporaneously, the role of CTs in the generation of mechanical allodynia in human subjects – a consistent theme that runs through the work presented in this thesis.

1. Review of classical mechanoreceptors

Tactile receptors in the skin are typically classified into *slow-adapting* (SA) and *fast-adapting* (FA) receptors according to their adaptive characteristics to ramp-and-hold indentations of the skin. The FA receptor displays action potential discharge only in response to a dynamic/changing event such as the onset and offset of a step indentation (or a vibratory stimulus), whereas the SA receptor maintains discharge during sustained indentation (Adrian, 1931; Iggo, 1977). It is worthy of note that these physiological differences have been attributed to distinct receptor morphologies mainly through post hoc anatomical correlations (e.g. Chambers *et al.*, 1972). Although direct evidence supporting many of these correlations is still lacking, recent molecular studies have established markers for different receptors (e.g. *Atoh1* for Merkel cells: Maricich *et al.*, 2009). However, most markers that are currently available label multiple receptor classes (Lumpkin *et al.*, 2010). Nonetheless, in future the advent of more selective markers, or a consensus review of their multiple expressions, may allow for an unabridged account of the morphology of receptors and their corresponding physiological functions.
1.1 Slow-adapting receptors

There are two distinct types of SA receptors, namely type I and type II. Although a third type of SA receptor was hypothesised in a human microneurography study (Edin, 2001), its existence remains questionable for want of compelling evidence. The type I SA receptors are associated with unencapsulated Merkel cells, which are located at the base of the epidermis in glabrous and hairy skin (Iggo & Muir, 1969; Jänig, 1971). The type II SA fibres are thought to arise from lightly encapsulated Ruffini endings, which are located in the dermis of hairy and glabrous skin as well as in joints (Goglia & Sklenska, 1969; Chambers et al., 1972). The SAII receptors are frequently encountered in the glabrous skin of the human, whereas their existence in the glabrous skin of the cat and monkey is less certain (Jänig et al., 1968; Knibestöl & Vallbo, 1970; Ferrington, 1985). What underlies this apparent discrepancy is not clearly known. Nonetheless, it has been shown that the innervation density of slow-adapting receptors in the monkey glabrous skin is much lower than the human glabrous skin (Talbot et al., 1968; Knibestöl & Vallbo, 1970). SAI receptors can be identified on the surface of hairy skin by dome-shaped protuberances termed touch spots that are identical to the Haarscheiben (hair discs) discovered by Pinkus (1902; see also Pinkus, 1964), albeit the association between touch domes and hairs varies across species (Iggo & Muir, 1969). In contrast, the SAI innervation of glabrous skin as well as the SAII innervation of hairy and glabrous skin are devoid of conspicuous surface features (Chambers & Iggo, 1967; Iggo, 1977). The SAI receptors have small, well-defined receptive fields with multiple zones of high sensitivity (Jänig et al., 1968; Johansson & Vallbo, 1979). Conversely, the SAII afferents have fairly large receptive fields with obscure borders, and a single zone of maximal sensitivity (Chambers et al., 1972; Johansson, 1978).
The morphological organisation of the SAI receptor is such that separate branches of the stem axon supply the individual Merkel discs, which may well explain the irregular firing pattern/inter-spike intervals during sustained pressure – a characteristic of SAI fibres (Iggo & Muir, 1969; Iggo, 1977; Wellnitz et al., 2010). In contrast, SAII fibres exhibit a characteristic regularity of discharge that seems consistent with the morphology of the Ruffini ending, wherein an undivided axon enters the capsule and bifurcates to form an intracapsular terminal arborisation (Chambers et al., 1972). However, the regularity of discharge disappears at low firing frequencies, which lends ambiguity to the differentiation between SAI and SAII responses. According to Wellnitz and colleagues (2010), this may account for the lack of SA afferent type discrimination, or the presumed absence of SAII fibres, in mouse hairy skin.

In terms of the functional significance, microstimulation of single SAI afferents in the glabrous skin generates a percept of sustained pressure (Ochoa & Torebjörk, 1983; Macefield et al., 1990). On the contrary, whether the SAI-fibre inputs from hairy skin are endowed with perceptual attributes remains to be elucidated (Harrington & Merzenich, 1970; Järvilehto et al., 1976). Furthermore, the SAI system has the capacity to provide information about the size, contour/curvature and force applied by the object indenting the skin (Ochoa & Torebjörk, 1983). As regards the SAII system, activation of individual afferents has consistently failed to evoke any sensation (Macefield et al., 1990). In light of their remarkable sensitivity to directional stretching and joint movement, the SAII afferents seem to have a functional role with relation to kinaesthesia and motor control (Knibestöl & Vallbo, 1970; Edin, 2001).
1.2 Fast-adapting receptors

Fast-adapting (FA) responses are associated with two encapsulated end organs, namely Meissner corpuscles (MC: type I) and Pacinian corpuscles (PC: type II). The former are exclusive to the dermis in glabrous skin, whereas the latter are found in the deeper (subdermal) layers of glabrous skin, but more remotely in the hairy skin such as in the vicinity of joints and interosseous membrane (Calne & Pallis, 1966; Iggo, 1977). Interestingly, the Meissner corpuscles are innervated by myelinated and unmyelinated afferents – first reported by Dogiel (1892). Furthermore, they appear to be functionally homologous to the Krause’s corpuscles in the cat (Iggo & Ogawa, 1977). Conversely, signals from the Pacinian corpuscles seem to be propagated mainly by afferents within the large-fibre range (Hunt & McIntyre, 1960). Akin to the SAI afferents, the FAI fibres have small, sharply defined receptive fields that, in part, reflects their superficial location within the skin. Following the same premise, the FAII receptors (just like the SAI afferents), which are located deep/remote to the skin, have large receptive fields with obscure borders (Johansson, 1978; Ochoa & Torebjörk, 1983).

Microstimulation of single fast-adapting afferents evokes sensation of tapping-flutter-vibration across a low-to-high frequency gradient. Interestingly, the percept of vibration evoked by activation of FAI or FAII afferents at high stimulation frequencies is qualitatively indiscernible (Ochoa & Torebjörk, 1983). However, it has been deduced from correlative studies in the monkey and human that the FAI system preferentially signals low-frequency mechanical events (less than ~50 Hz), thereby eliciting a percept of tapping-flutter. Conversely, the FAII system
preferentially signals high-frequency events (more than ~50 Hz), hence generating a percept of vibration (Talbot et al., 1968).

The skin of the face exhibits a lower sensitivity to high-frequency vibration relative to the glabrous skin of the distal limbs. This is consistent with evidence for the FAII innervation of the face (Barlow, 1987; Johansson et al., 1988). However, the incongruity in subjective thresholds for vibration detection may, in part, be a result of differential proximity of PC units in the two regions. Adrian and Umrah (1929) postulated that the PC units are responsible for mediating static pressure and, to some extent, joint movement; a hypothesis at least partly based on the morphology of the Pacinian corpuscles and their anatomical pattern of distribution. However, an interconversion between pressure and tapping-flutter-vibration has not been observed in microneurography studies involving selective activation of FAII afferents (Ochoa & Torebjörk, 1983). For comparison, see Vallbo (1981). In terms of the perceptual outcome, intraneural microstimulation is not identical to natural stimulation of the skin; the former evokes activity in a single unit, whereas the latter triggers activity in a varied population of receptors (Ochoa & Torebjörk, 1983). This suggests that a gamut of multiple elementary sensations underlie our perceptual response to a given stimulus.

Hair follicle afferents (HFA) constitute another kind of fast-adapting mechanoreceptors that, as the name implies, are found exclusively in hairy skin. Within the HFA class, several different types of hair units have been discovered across a range of species and regions of skin (Iggo, 1977). Hair units with myelinated fibres discharge impulses during displacements of the individual hairs, and when
they are returning to their original positions (Vallbo et al., 1995). In addition, correlative studies in the monkey and human have shown that the HFA respond vigorously to vibration, particularly in the low frequency range (Merzenich & Harrington, 1969; see also Mahns et al., 2006). The receptive fields of hair units are large and oval (or irregular) in shape with multiple, closely-packed spots of high sensitivity that correspond to individual hairs (Vallbo et al., 1995). However, the size of the receptive field is considerably smaller at the margin of glabrous and hairy skin (Iggo, 1977). Psychophysical studies have shown that the HFA and FAII in the hairy skin are functionally equivalent to their FAI and FAII counterparts in the glabrous skin (Talbot et al., 1968; Merzenich & Harrington, 1969). However, contrary to the glabrous skin, the extent to which the sensations of tapping-flutter-vibration can be attributed to distinct neural substrates in the hairy skin is yet to be elucidated.

Recently, Li and colleagues (2011) demonstrated using genetic markers that each hair type is innervated by an ‘invariant’ combination of low-threshold mechanoreceptors. For example, the guard hairs – located in the trunk hairy skin of mice – are associated with RA and SA myelinated afferents, but not the unmyelinated mechanoreceptors. Whether this innervation pattern can be extrapolated to the guard hairs that form the human scalp hairs, or the outer long hairs of other furry animals, is unknown. It was originally thought that the longitudinal lanceolate endings associated with hair follicles are exclusively the domain of myelinated afferents. However, we now know that unmyelinated mechanoreceptors form longitudinal lanceolate endings in association with zigzag and awl/auchene hair follicles on the back hairy skin of mice (Li et al., 2011). Such advances in molecular/genetic strategies challenge the presumptuous notion that free
nerve endings, within the C-fibre range, are limited to pain and temperature substrates/sensations (e.g. Munger & Ide, 1988; Halata, 1993) that implies little or no regard for the abundant C mechanoreceptors.

Field units constitute another kind of fast-adapting receptors that are innervated by large myelinated fibres (Leem *et al.*, 1993). Whether their innervation profile is limited to a particular large-fibre class, or constitutes myriad and disparate afferent classes, is yet to be elucidated. The receptive fields of field units are fairly similar to the hair units, although the individual high-sensitivity spots are larger and more closely packed than those of the hair units (Vallbo *et al.*, 1995). Furthermore, the receptive fields are preferentially excited by direct low-threshold contact with the skin than by hair movement (Light & Perl, 1979). Presumably a subclass of field units are activated by cooling as well, albeit whether the resulting afferent discharge contributes to thermal sensibility is unclear (Booth & Hahn, 1974). The absence of a detailed characterisation of field receptors limits the determination of their functionality.

### 2. Peripheral origins of vibrotactile sensibility

Sinusoidal vibration has been extensively used in tactile research (e.g. Hunt, 1961; Sahai *et al.*, 2006), given the capacity for generating a reproducible stimulus with precisely defined parameters (frequency, amplitude and duration). In addition, it allows for a differential activation of mechanoreceptors: within the bandwidth of human vibrotactile sensibility (~5-1000 Hz), lower frequencies (less than ~50 Hz) are mediated by HFA in hairy skin and FAI in glabrous skin, whereas higher frequencies (greater than ~50 Hz) are signalled by FAII afferents in both skin types.
Given that the PC units are not located in the hairy skin, but in the vicinity of joints and interosseous membrane, their capacity to signal vibrotactile stimuli, applied to the skin, can be attributed to a highly specialised structure for mechanotransduction, in addition to the viscoelastic properties of the surrounding tissues (Moore, 1970; Munger & Ide, 1987; Mahns et al., 2006).

In addition to the fast-adapting afferents, the slow-adapting fibres in both skin types respond to vibration, particularly in the low frequency range. However, it is often argued that they do not contribute to the vibration sense, given the discord between the thresholds for vibrotactile detection and the entrainment of SA afferents by vibratory stimuli. Furthermore, at vibration frequencies above 20 Hz, the SA afferents are less sensitive compared with the RA afferents, which makes the contribution of the former to vibrotactile sensibility exceedingly unlikely (Talbot et al., 1968; Merzenich & Harrington, 1969; Mahns et al., 2006). Not to mention, microneurography studies have consistently shown that activation of single SA afferents does not yield a vibrotactile percept (as discussed above). Notably, the perceptual outcome (if any) of single-unit activation of low-threshold myelinated afferents, regardless of the stimulus frequency, is invariably and unequivocally innocuous (Ochoa & Torebjörk, 1983; Vallbo et al., 1984; Macefield et al., 1990).

3. Low-threshold unmyelinated fibres

The existence of C low-threshold mechanoreceptors (CLTMRs) was first reported by Zotterman (1939) in the skin of cat. Over the years, the existence of CLTMRs has been confirmed in a numbers of studies on non-human animals (Maruhashi et al.,
1952; Douglas & Ritchie, 1957; Iggo, 1960; Bessou et al., 1971; Kumazawa & Perl, 1977; Shea & Perl, 1985). However, it was suggested that the CLTMR system in humans constituted an evolutionary vestige based on the finding of a dwindling representation along the evolutionary timeline from cat to monkey (Kumazawa & Perl, 1977). Furthermore, an apparent absence of evidence using the microneurography technique in awake human subjects – that persisted until the findings of Johansson and colleagues (1988) – lent credence to this hypothesis, particularly in light of the ongoing characterisation of a range of nociceptive C-fibre types (Torebjörk, 1974; Torebjörk & Hallin, 1974; Ochoa & Torebjörk, 1989; see also Schmidt et al., 1995). Moreover, psychophysical and microneurography studies have unequivocally demonstrated the dependence of tactile sensibility (detection/discrimination/localization) on intact myelinated fibres (Sinclair & Hinshaw, 1950; Torebjörk & Hallin, 1973; Mackenzie et al., 1975; Hallin & Torebjörk, 1976; Dellon, 1980). We now know that the human hairy skin is densely innervated by a class of C fibres that responds to gentle touch. In fact, it has been reported in recent microneurography studies that these fibres are encountered almost as frequently as the myelinated fibres (Vallbo et al., 1993; Löken et al., 2009).

The initial affirmation of the existence of CTs came from Johansson et al. (1988), which was followed by a more detailed description by Nordin (1990). To date, CT-fibre recordings, using microneurography, have been performed on the following human nerves: supra- and infra-orbital; lateral cutaneous femoral; lateral and dorsal antebibrachial cutaneous; superficial radial; and common/superficial peroneal (Johansson et al., 1988; Nordin, 1990; Vallbo et al., 1993; Serra et al., 1999; Vallbo
et al., 1999; Edin, 2001). These findings may well denote the ubiquitous distribution of CTs in human hairy skin.

Currently, there is no evidence for the existence of a phenotypically identical class of CTs in human glabrous skin (as detailed in the following sections). Whether a functional homologue of CTs, much like the HFA and FAI in hairy and glabrous skin respectively, exists in the glabrous skin remains untested. It is yet to be ascertained whether the ‘absence of evidence’ truly represents a lack of CT-fibre innervation in the glabrous skin, or can it be attributed to the following: differences across skin types in terms of receptor characteristics and biophysical properties (Montagna & Parakkal, 1974; Quilliam, 1978; Boada et al., 2010); and a reduction in innervation density along a proximal-to-distal gradient (Kumazawa & Perl, 1977; Liu et al., 2007; Li et al., 2011) combined with the increased likelihood of pronounced masking effect of sympathetic efferent activity in the distal regions (Löken et al., 2007). Based on our observations, the presence of a functionally equivalent class to the CTs in the glabrous skin is highly plausible. This has been discussed in detail in Paper II and Section 3.2 of this thesis. Whether C mechanoreceptors innervate the genitalia is unclear. The ambiguity, in part, arises from the use of molecular/genetic markers that express with considerable variability, in addition to a tendency to readily ascribe a ‘definitive’ marker to this class of afferent (see Section 3.2 for details).

3.1 Receptor characteristics

Based on a phylogenetic classification, C mechanoreceptors represent a very ancient system of slowly conducting unmyelinated afferents (Zotterman, 1939). Their conduction velocity is about 1 m s⁻¹, which seems fairly consistent across a range of
species, including rats, cats and humans (Roberts & Elardo, 1985; Vallbo et al., 1999; Zhang et al., 2008). The individual receptive field of a CT fibre, mapped using skin indentation, consists of several hot spots interspaced with less responsive regions (Wessberg et al., 2003). The axonal branches display extensive arborisation, and form longitudinal lanceolate endings that have been observed in close association with hair follicles (Li et al., 2011). The receptive fields are approximately round or oval without any preferred orientation (Wessberg et al., 2003). Furthermore, the receptive terminals display sensitivity to both innocuous and noxious stimuli, albeit the response properties have been tested using a limited range of stimuli. Examples include finger stroking, soft brushing, skin stretch, skin indentation and pinprick (Vallbo et al., 1993; Vallbo et al., 1999; Edin, 2001; Löken et al., 2009). The receptive field size varies according to the intensity of stimulation (1-35 mm² at 5 mN indentation force); a property attributed to the sensitivity of CTs to skin stretch (Kumazawa & Perl, 1977; Wessberg et al., 2003). However, this link may depend upon whether the receptive field is located in high-compliance (e.g. over muscle) or low-compliance (e.g. over bone) tissue (Moore, 1970; Slugg et al., 2000).

CT fibres have a predilection for slowly moving, low-force mechanical stimuli such as soft brushing (Löken et al., 2009). The activation of CTs using brushing has been reported in animal studies as well (Seal et al., 2009; Andrew, 2010). In order to distinguish CT fibres from their nociceptive counterparts, thresholds to skin indentation are often used – a monofilament threshold below 5 mN is attributed to a CT fibre, whereas a threshold above 5 mN is classified as a C nociceptor (Löken et al., 2009). It has been shown in microneurography recordings that a CT fibre does not respond differentially during innocuous and noxious (mechanical) stimulation,
whereas a C nociceptor responds preferentially to a noxious stimulus (Vallbo et al., 1999). However, recent animal work has demonstrated that noxious mechanical stimulation produces a significantly greater response in CLTMRs than innocuous stimulation, thereby implicating CT fibres in mechanical nociception (Seal et al., 2009). If CT fibres do indeed contribute to pain, then it calls into question the boundary between classical nociceptors and tactile fibres. Whether the two classes contribute to qualitatively different perceptual and emotional states warrants exploration. This question is, in part, the focus of the work presented here.

During sustained indentation, the ‘typical’ response of a CT fibre involves an initial high frequency burst of impulses (up to 100 per s), which is followed by a gradually falling frequency that may abate to near silence. The adaptive properties of CTs seem intermediate to the response patterns of fast-adapting and the slow-adapting fibres during long-lasting indentation (Iggo, 1960; Olausson et al., 2010). However, in a subset of CTs, the initial phase of adaptation is followed by a recovery of the discharge frequency, a phenomenon known as delayed acceleration, which was attributed to these afferents by Vallbo and colleagues (1999). There is paucity of information about the characteristics of CTs that exhibit this feature, particularly whether their sensitivity to various stimuli changes during the resurgent phase. It has been proposed that the CT-fibre discharge – during delayed acceleration or otherwise – does not have immediate/direct access to conscious awareness, given the absence of any overt sensation (Vallbo et al., 1999; McGlone & Reilly, 2010). In some C units, the discharge of impulses outlasts the stimulus duration, albeit at a reduced impulse rate. This phenomenon is called after-discharge, and was first reported by Zotterman (1939). Subsequent studies have confirmed the presence of such responses
(Iggo, 1960; Bessou et al., 1971; Kumazawa & Perl, 1977), which appears to be a unique property of C fibres (Hensel et al., 1960). Furthermore, after-discharge is more frequently observed following slow, gentle stroking of the skin (Iggo, 1960; Nordin, 1990), or when the site of mechanical stimulation is cooled (Wiklund Fernström, 2004). In addition, the expression of after-discharge depends, in part at least, on the interval between successive stimuli. For example, mechanical stimulation following an interval of a few minutes resulted in a greater duration and extent of after-discharge relative to an interval of just a few seconds (Kumazawa & Perl, 1977).

In the cat, C mechanoreceptors respond to a gentle ruffle of the hair with a feather or a puff of air (Zotterman, 1939; Iggo, 1960; Hahn, 1971). However, the responsiveness of this fibre class to hair movements is not as robust as the mechanosensitive myelinated fibres. Therefore, it has been suggested that a C mechanoreceptor is not a typical hair receptor (Bessou et al., 1971). In humans, the sensitivity of CT fibres to hair movements has not been explored in detail (Olausson et al., 2010). Nonetheless, it appears that the CTs are responsive to the movement of a group of hairs, or single hairs for that matter, but not to the sustained displacement of hairs (Nordin, 1990).

There is a dearth of studies that test the responsiveness of CT fibres to rapidly moving stimuli. Bessou and colleagues (1971) proposed that CLTMRs in the cat have limited responsiveness to rapidly moving stimuli. Interestingly, myelinated mechanoreceptors and CLTMRs have overlapping thresholds for mechanical stimulation (Hensel et al., 1960; Iggo, 1960). Furthermore, the responsiveness of
CLTMRs increases in parallel to the amplitude of skin indentation, a property shared with myelinated mechanoreceptors (Iggo & Kornhuber, 1968). However, a change in indentation rate has no effect on the peak discharge rates of C mechanoreceptors (Bessou et al., 1971). This may denote a suboptimal capacity of CT fibres to code for the discriminative aspects of touch. CT-responsiveness to rapidly moving stimuli depends, in part at least, on the interval between successive stimuli, given that a progressive fall, or ‘fatigue’, in the response elicited when the inter-stimulus interval was reduced to less than 30 s (Iggo, 1960; Bessou et al., 1971; Kumazawa & Perl, 1977). In healthy human subjects, Vallbo and colleagues (1999) demonstrated that the initial spike activity in CTs remained fairly consistent during repetitive stimulation; a finding suggesting a reduced susceptibility of CTs to fatigue in humans. In recent years, functional subtypes of C fibres have been distinguished based on their patterns of activity-dependent conduction slowing (Serra et al., 1999; Obreja et al., 2010). Based on that, CT fibres were linked to a ‘type 3’ profile, which denotes a resistance to use-dependent inactivation, as displayed by only a slight increase in latency during repetitive stimulation – electrical as well as concomitant mechanical. In contrast, C nociceptors displayed a profound change, whereby the conduction slowing progressed to the point of inactivation in many units (Serra et al., 1999; Campero et al., 2011). However, the functional significance of the resistance of presumed CTs to use-dependent inactivation is not yet fully elucidated.

It is important to interpret the characteristics of C fibres in light of the broader heterogeneity within this afferent class, and the variance across species. For example, cold units in primates, including humans, contain small myelinated axons (Hensel & Boman, 1960; Iggo, 1969), while their functional counterparts in sub-primates are
mainly subserved by C fibres (Hensel et al., 1960). There have been no studies conducted on healthy human subjects to quantitatively examine the response properties of CT fibres to rapidly moving stimuli such as vibration, let alone whether vibration-evoked C-fibre discharge correlates with a perceptual response. We do, however, know that the recovery time for CT-fibre fatigue is about 30 s in humans (Wiklund Fernström, 2004) that befits the stimulation intervals adopted in recent psychophysical and microneurography studies (e.g. Løken et al., 2009), including the work presented in this thesis.

The application of vibration in a patient with sensory neuronopathy syndrome, which is postulated to be a disorder of nerve cell bodies of large primary sensory neurons, revealed an impaired capacity for detection (Sterman et al., 1980; Olausson et al., 2002). This observation is consistent with the conventional understanding whereby vibrotactile sensibility is the exclusive function of large myelinated afferents. Although little is known about the small myelinated mechanoreceptors (Aδ), their capacity to respond to vibrotactile stimulation has been demonstrated (Merzenich & Harrington, 1969; see also Adriaensen et al., 1983). However, the cool detection threshold in this particular patient was significantly elevated, which was taken as being indicative of at least a partial defect in the Aδ-fibre system (Olausson et al., 2002). In a later publication by Olausson and colleagues (2008a), however, the cool detection threshold was reported within normal range. More importantly, a lack of perceptual response does not preclude the activation of CT fibres. For example, C mechanoreceptors are excited by rapid cooling of the skin (Douglas & Ritchie, 1957; Iggo, 1960; Bessou et al., 1971; Kumazawa & Perl, 1977), but that activity seemingly plays no role in thermal sensibility (Mackenzie et al., 1975; Yarnitsky &
Ochoa, 1991). This property of CLTMRs is consistent with the large myelinated slow-adapting mechanoreceptors that were termed ‘spurious’ thermoreceptors by Iggo (1969). However, the discharge frequency of both afferent classes during rapid cooling is appreciably smaller than the activity triggered by mechanical stimulation (Bessou et al., 1971; Kumazawa & Perl, 1977).

Mechanical-thermal interactions, when the two stimuli are applied concurrently, remain poorly understood, and invite speculation about different encoding mechanisms that underlie the resulting signals. For example, concurrent punctate mechano-thermal stimulation results in summation, albeit incomplete, of the responses to the individual stimuli, while a somewhat inhibitory effect on the CT-fibre activity has been observed when moving mechano-thermal stimuli are applied (Hahn, 1971; Liljencrantz et al., 2010). In this context, it might be interesting to revisit Weber’s deception, the perception of increased weight of cooled objects, given that it has only been looked at with respect to the large mechanoreceptors that respond to cooling, and not C mechanoreceptors (Weber, 1851; Hensel & Zotterman, 1951). It’s tempting to suggest that the latter contribute to the percept of pressure during cooling, particularly given that Kiesow (1911) evoked a pressure sensation by evaporation of ether, which has been shown to activate CT fibres (Nordin, 1990).

Based on microneurography studies in humans, CT fibres do not respond to warming or noxious heating (Vallbo et al., 1999). This is consistent with observations in the monkey where little or no response elicited during skin heating (Kumazawa & Perl, 1977). However, at evidently damaging temperatures, a transient response has been reported in the cat, which appears to be of little functional significance in normal
conditions (Iggo, 1960). Interestingly, rapid innocuous warming excited a few CLMTRs innervating the ear skin of the rabbit (Shea & Perl, 1985). Whether this observation can be extrapolated to the human ears remains untested.

### 3.2 Chemical phenotype

Despite a recent explosion in the use of molecular/genetic techniques, we know very little about the chemical phenotype of CLTMRs largely due to the varying/limited target specificities of the existing markers. Nonetheless, the molecular markers do distinguish between myelinated fibres, CLTMRs and peptidergic nociceptors. CLTMRs do not express neurofilament H (NFH), which is a marker for myelinated fibres. Furthermore, they do not express calcitonin gene-related polypeptide (CGRP) and transient receptor potential vanilloid 1 (TRPV1) that are markers for peptidergic nociceptors (Albers et al., 2006; Liu et al., 2007; Seal et al., 2009; Li et al., 2011). The latter is consistent with the lack of CT-fibre responses to noxious heat and capsaicin in humans (Vallbo et al., 1999; Wiklund Fernström, 2004). In contrast, it is possible, albeit untested, that cold-sensing channels such as TRPM8 are expressed in CLTMRs, given their sensitivity to cooling of the skin (Peier et al., 2002).

It has been shown that the tyrosine hydroxylase-labelled (TH-positive) CLTMRs express the nonpeptidergic nociceptor markers c-RET and glial cell line-derived neurotrophic factor family receptor α2 (GFRα2), but do not bind/express isolectin B4 (IB4) as well as Mas-related G protein-coupled receptors (Mrgprs). The latter observation is intriguing, particularly in light of the findings of Liu and colleagues (2007) who proposed the MrgprB4-positive neurons as a marker of CLTMRs. In contrast to TH-positive neurons, MrgprB4-positive neurons were found to be IB4-
positive, and *unresponsive* to mechanical and thermal stimuli – key response characteristics of CLTMRs. Moreover, MrgprB4-positive fibres were not detected in the glabrous skin and the genitalia, which was interpreted as the absence of CLTMRs from these regions. This assertion must be questioned on two accounts: the mismatch between the expression of MrgprB4-positive fibres and the known response properties of CLTMRs; and reports that other family members of Mrgprrs are widely expressed within glabrous skin (e.g. Zylka *et al.*, 2005). Furthermore, the sacral dorsal root ganglion (DRG) neurons that innervate the genitalia have a sizable population of TH-positive neurons, which makes the presence of CLTMRs in the genitalia quite likely (Drake *et al.*, 2005; Li *et al.*, 2011). As regards the glabrous skin of the distal limbs, on the one hand TH-positive neurons were not found in the DRG that innervate glabrous region, but on the other hand more than three-fourths of the TH-positive neurons expressed the vesicular glutamate transporter VGLUT3, which is found in a subset of DRG cells that innervate the glabrous skin (Seal *et al.*, 2009; Li *et al.*, 2011). The apparent ambiguity in the literature can, in part, be attributed to the variability in the expression of CLTMR markers amongst the DRG neurons, which ranges from under 2% to about 30% (Liu *et al.*, 2007; Seal *et al.*, 2009; Li *et al.*, 2011).

Zhang and colleagues (2008) have recently proposed that the conduct of CLTMRs is modulated by neighbouring peptidergic afferents through the release of Substance P (SP) that binds to Neurokinin-1 (NK-1) receptors located on CLTMRs. The authors suggest that this peripheral *cross-talk* points toward the role of CLTMRs in pain processing, particularly in allodynia. Seal and colleagues (2009) have also implicated CLTMRs in pain processing (*see* Sections 3.3 & 3.5) by demonstrating the
attenuation of mechanical hypersensitivity in the glabrous skin of VGLUT3-knockout mice (although VGLUT3-positive neurons do not express SP). Furthermore, it has been shown that mechanical allodynia – evoked in glabrous skin – can be abolished by subcutaneous application of an NK-1 antagonist (Carlton et al., 1996). Therefore, converging evidence connotes the possible existence of CLTMRs in glabrous skin, in addition to their role in mediating allodynia. The latter proposition need not preclude the contribution of large myelinated and unmyelinated polymodal neurons, including the upregulation of inflammatory mediators and their corresponding receptors, to the pain-states (e.g. Schaible et al., 2002).

It might be interesting to revisit another member of the MrgrpRs, called the MrgrpD, in the context of CLMTRs. The MrgrpD-expressing neurons are found exclusively in the skin – hairy and glabrous. Consistent with the expression of the known CLTMR markers (discussed above), MrgrpD-expressing fibres are negative for the peptidergic nociceptor markers CGRP and TRPV1 (Zylka et al., 2005; Liu et al., 2007; Seal et al., 2009; Li et al., 2011). Akin to the TH-positive and MrgrpB4-positive neurons, they express the nonpeptidergic nociceptor marker c-RET (Zylka et al., 2005; Liu et al., 2007; Li et al., 2011). In addition, they bind the lectin IB4, which is consistent with MrgrpB4-positive neurons (Zylka et al., 2005; Liu et al., 2007). Moreover, they are positive for peripherin – a marker for nociceptors with unmyelinated axons – that VGLUT3-positive neurons express as well (Zylka et al., 2005; Seal et al., 2009). However, contrary to the MrgrpB4-positive neurons, most of the MrgrpD-expressing fibres were found to be positive for purinergic receptor X3 (P2X3). In terms of the pattern of axonal endings in the skin, MrgrpD expression was not detected in guard hairs, which is augmented by reports on an absent innervation
of this hair follicle type by TH-positive fibres (Zylka et al., 2005; Li et al., 2011). However, TH-positive neurons were detected in close association with zigzag and awl/auchene hair follicles (Li et al., 2011). The CGRP-positive (peptidergic) and MrgprD-positive (nonpeptidergic) neurons are often intimately intertwined in the glabrous skin, which can be mistaken for a single subtype with an overlapping molecular expression (Zylka et al., 2005). This observation signifies the difficulties in identifying the C-fibre substrates in glabrous skin.

Epidermal keratinocytes have been proposed as the preferential detectors of stimuli applied to the skin surface, which in turn convey the information to the epidermal free nerve endings (Denda et al., 2007). Given that MrgprD-positive fibres project exclusively to the epidermis, they may contribute to such cross-talk and, by implication, to skin surface perception. Whether CLTMRs express MrgprD remains a matter for conjecture. However, in case of the affirmative, given the close association between peptidergic and nonpeptidergic fibres in the skin, this will be consistent with the hypothesis put forth by Zhang and colleagues (2008) entailing the peripheral modulation of CLTMRs by neighbouring peptidergic fibres.

In addition to SP, as proposed by Zhang and colleagues (2008), a range of other mediators may be involved in mediating the cross-talk following nerve injury and inflammation. It has been shown that CLTMRs are sensitive to histamine, which also led to the suggestion of their role in mediating itch (Lynn, 1992). Glutamate may be another candidate involved in peripheral regulation, particularly given that the CLTMRs express glutamate receptors (Cao et al., 2007). Furthermore, glutamate interacts with NK-1 receptors as well, which seem to be located on CLTMRs.
(Carlton et al., 1998; Zhang et al., 2008). Notably, mechanical allodynia – evoked in glabrous skin – can be blocked by peripheral application of glutamate antagonists (Carlton et al., 1995; Carlton et al., 1998). In addition to the various inflammatory mediators/receptor systems described above, CLTMRs are excited, albeit to a variable degree, by sympathetic stimulation, and vice versa. This interrelation has been observed across a range of species, including rabbits, cats and humans (Roberts & Elardo, 1985; Barasi & Lynn, 1986; Olausson et al., 2008a). However, its functional significance is largely unexplored. It is tempting to suggest that the CT fibres may be involved in sympathetic-afferent interactions that underlie the sustenance, or expression, of certain neurological disorders such as complex regional pain syndrome (Jänig & Baron, 2003; Wasner et al., 2003; Baron, 2006).

3.3 Spinal projections

The superficial layers of dorsal horn appear to be the main projection zone for CLTMRs from hairy and glabrous skin (Keller et al., 2007; Seal et al., 2009; Andrew, 2010). Using a plant lectin (Phaseolus vulgaris leucoagglutinin) to mark functionally defined C fibres, Sugiura and colleagues (1986) showed that the CLTMRs terminate in outer part as well as the inner part of lamina II of dorsal horn. In addition, the MrgprB4-expressing neurons, which seemingly represent CLTMRs, terminate in the outer part of lamina II (Liu et al., 2007). Using VGLUT3 as a marker, it has been shown that CLTMRs terminate in laminae I and innermost II (Seal et al., 2009). Furthermore, VGLUT3-labelled fibres innervate the interneurons that express protein kinase C gamma (PKCγ). Notably, PKCγ has been deemed essential for the generation of injury-induced mechanical allodynia (Malmberg et al., 1997). Furthermore, the PKCγ interneurons express Fos in response to innocuous,
but not noxious, stimulation (Neumann et al., 2008). It is deducible by inference that
the functional peculiarity of the lamina II-inner neurons stems from their capacity to
differentially modulate low-threshold inputs, such as a crossover from touch to pain
during inflammation or injury. Given that large myelinated fibres terminate in the
inner part of lamina II, in addition to lamina III/IV, the functional significance of two
distinct low-threshold inputs to this region is unclear. Drew and MacDermott (2009)
conjectured that the dual innervation of the inner part of lamina II may allow for a
CLTMR-driven modulation of large-fibre tactile input under injury conditions. Such
a mechanism may underlie pain-induced perceptual and behavioural adjustments, in
addition to providing spatial cues during tactile stimulation. However, co-activation
of myelinated and unmyelinated fibre classes need not be required to elicit sensory-
perceptual realignments during pain-states (see following text).

Recently, Seal and colleagues (2009) proposed that VGLUT3-labelled CLTMRs
contribute to the expression, but not the induction, of central sensitization. What
provided credence to this proposition was the differential character of responses in
the setting of inflammation/injury/trauma, wherein mechanical hypersensitivity was
diminished in the VGLUT3-knockout mice, yet their responses to heat
(hypersensitivity) were comparable to the control animals. Furthermore, based on
their observations, CLTMRs appear to have direct access to ‘pain’ pathways,
particularly given the acute nature of capsaicin-induced interactions. Intriguingly, in
the absence of any pain-inducing manipulations, these knockout mice exhibited a
subtle defect in their responses to intense noxious mechanical stimuli, thereby
implicating the CLTMRs in acute mechanical pain sensation. However, the role of
classical nociceptors is no less pertinent to central sensitization and pain perception.
The superficial laminae constitute a major termination region for the classical nociceptors (Light et al., 1979; Todd et al., 2003). In addition, the role of nociceptive input seems pivotal in realising an altered integration and processing of information following inflammation/injury (Foreman et al., 1979; Woolf & Salter, 2000).

Andrew (2010) recently showed that the CLTMR-mediated input projects from lamina I spinoparabrachial neurons to parabrachial nucleus in the brainstem of rat. However, the circuitry involved in conveying information from lamina II neurons (where CLTMRs terminate) to lamina I projection neurons is yet to be ascertained. In comparison to the spinoparabrachial pathway in the rat, it is plausible that the CT information in the human is relayed by the lamina I spinothalamic-ventromedial thalamic nucleus (posterior portion, VMpo) pathway. Inputs from the VMpo project to the dorsal posterior insular cortex (Craig, 2002).

### 3.4 Cortical projections

Functional imaging studies in two patients with loss of large-fibre function have shown the activation of posterior insula during gentle brushing of the skin (Olausson et al., 2002; Olausson et al., 2008b). Notably, the somatotopic organisation displayed by posterior insula for the processing of soft brushing in these patients is remarkably similar to that reported for cutaneous and muscle pain in healthy individuals (Henderson et al., 2007; Björnsdotter et al., 2009). However, the warmth thresholds of these patients were reported outside the normal range of detection, which indicates at least a partial impairment of the C-fibre system (Cole et al., 2006; Olausson et al., 2008a). Furthermore, the activation of the Aδ-fibre system by gentle brushing warrants further exploration, given that the cool threshold (Aδ-mediated), in one of
the two patients, appears to be within a normal range of detection (Adriaensen et al., 1983; Olausson et al., 2002; Olausson et al., 2008a). Nonetheless, the activation of posterior insula by brush stroking has been reported in healthy individuals as well (Gordon et al., 2011). The activation of posterior insular cortex during highly resolved feelings such as pain and emotional/affective touch is consistent with the proposed role of this region in interoception (Craig, 2003). In addition to the posterior insula, the orbitofrontal and anterior cingulate cortices are activated during pleasant and painful tactile stimuli applied to hairy and glabrous skin (Francis et al., 1999; Rolls et al., 2003; Gordon et al., 2011). In contrast, Olausson and colleagues (Olausson et al., 2008b) showed that the somatosensory cortices are not activated by gentle brushing in large-fibre deafferented patients. However, this observation should be treated with some caution given the findings on extensive thinning of the cortex in these patients (personal communication; see also Čeko et al., 2012). It has been reported that “selective CT-stimulation fails to evoke anything like a full sensation of pleasant touch” in these patients (McGlone et al., 2007). Whether this effect can be attributed to the differential activation of limbic-emotional regions vis-a-vis discriminative-cognitive areas is unclear.

3.5 Functional significance

It has long been recognised that the behaviour of CLTMRs peripherally and their presumed effects centrally distinguish them from their myelinated counterparts. However, the functional role of these receptors has remained elusive. Zotterman (1939) postulated that CLTMRs subserve a sensation of tickle. The following points denote the rationale behind Zotterman’s tickle hypothesis: the capacity of gentle hair movements to stir activity in CLTMRs – an effect that was reduced, or abolished,
when the cat fur was moistened with water or Ringer’s solution; and a comparable experience in everyday life where gentle stroking of the hairs elicits a tickling (or itching) sensation that disappears when the skin is moistened (Zotterman, 1939). Interestingly, in a large-fibre deafferented patient, gentle brushing of the hairy skin was described as a faint and diffuse sense of pressure that was clearly pleasant – not tickling (Olausson et al., 2002). However, the sensation of tickle is often associated with a pleasant feeling, akin to a fine feather stroking the skin (Ochoa & Torebjörk, 1983). In addition to the tickle and pleasant-touch hypotheses, Lynn (1992) put forward a supposal that CLTMRs mediate itch, which was founded on the sensitivity of these receptors to histamine. There is no psychophysical evidence to support this idea, but the pleasant feeling of scratching a nagging itch is a common observation nevertheless.

While electrically stimulating the unitary mechanosensitive afferents, Vallbo (1981) noted that touch, pressure, vibration and tickle are not invariant sensations, but display interconversion and at least partial concomitance in occurrence (cf. Ochoa & Torebjörk, 1983). Although this observation was limited to the myelinated afferents, it invites speculation that the ‘qualia’ attributed to CLTMRs represent a sensation continuum. The variations may be an effect of contextual biases and/or experimental parameters. For example, in the case of FAI stimulation, a tapping-to-flutter-to-vibration pattern of interconversion elicits along a low-to-high frequency gradient (Ochoa & Torebjörk, 1983). However, Douglas and Ritchie (1957) proposed that CLTMRs may be more concerned with sub-perceptual processing – an opinion shared by the protagonists of the pleasant-touch hypothesis (McGlone et al., 2007; Olausson et al., 2010). Roberts and Elardo (1985) proposed that the CLTMRs are
primarily involved in monitoring the physiological condition of the cutaneous tissues. The notion that CLTMRs play an essential role in interoception reverberates across a number of recent reviews (Craig, 2002; Björnsdotter et al., 2010; Morrison et al., 2010).

The pleasant-touch hypothesis is largely based on two patients with total dysfunction of large myelinated fibres. Furthermore, based on their cool and warmth detection values, at least a partial impairment of small myelinated and unmyelinated fibres is probable (Olausson et al., 2002; Olausson et al., 2008a). During gentle brushing of the hairy skin, the subjective report varied from “no sensations at all” to “a very weak, vague, and inconsistent” sensation of light touch in these patients (Olausson et al., 2010). Furthermore, intra- and inter-subject variances in the perceptual outcomes of CT stimulation were also noted. Notably, the sensation, if and when evoked, was “slightly or moderately pleasant with no hint of pain, tickle, or itch” (Olausson et al., 2010). In healthy subjects, it was recently shown that CTs display an inverted U-shaped (negative quadratic) response curve to graded brushing velocities with peak discharge occurring at 1-10 cm s\(^{-1}\). Conversely, myelinated afferents exhibited a characteristic linearity between discharge and velocity. Interestingly, while all sensory fibres were intact, subjective ratings of perceived pleasantness followed an inverted U-shaped pattern in relation to brushing velocity. Based on correlation between neural discharge (impulse per s) and perception, Löken and colleagues (2009) concluded that CTs mediate pleasant-touch. However, as discussed in Paper I, an alternative explanation can be advanced, namely that CTs and myelinated afferents are activated in parallel during brushing with a sensation of pleasantness emerging when the responsiveness of myelinated afferents exceeds
that of CT fibres. In contrast, at the lowest brushing velocities, neutral or unpleasant (negative pleasant) ratings were reported where the responsiveness of CT fibres may well exceed that of myelinated afferents. This signifies the merit of examining the role of CTs beyond the pleasant-touch domain.

Certain convergent, albeit ancillary, observations in the literature raise the possibility of **CT-functionality in ‘pain’ context.** For example, in one of the two large-fibre deafferented subjects described above, an otherwise innocuous monofilament (40 mN) – applied to the untreated skin – was perceived as sharp following a capsaicin injection (Treede & Cole, 1993; Cole et al., 2006). Although this effect was ascribed to small-fibre (Aδ and C) nociceptors, it is tempting to suggest that the role of CT fibres may be crucial in such interactions, not least because of their capacity to encode punctate stimuli (Vallbo et al., 1993; Cole et al., 2006). Following the same premise, Maruhashi and colleagues (1952) noted a remarkable level of after-discharge in CLTMRs of the cat following mechanical stimulation of a scalded region of skin. More recently, Zhang and colleagues (2008) proposed that CLTMRs of the rat may contribute to tactile allodynia in a state of peripheral sensitization – an effect purportedly regulated by peptidergic nociceptors through SP and NK-1 receptors (as discussed in Section 3.2). Perhaps the most convincing data thus far on the role of CLTMRs in pain processing has been provided by Seal and colleagues (2009). Mice lacking the VGLUT3-labelled functionally defined CLTMRs displayed small defects in the generation of mechanical pain such as a delayed response/increased threshold to intense noxious mechanical stimuli. Furthermore, during inflammatory or neuropathic ‘pain’ conditions, stimulation of the plantar hindpaw failed to elicit mechanical hypersensitivity (alldynia) in the knockout mice.
These observations underscore the critical contribution of CLTMRs to pain sensation in general, and allodynia in particular. However, this need not preclude the role of large myelinated (Aβ) afferents and C polymodal nociceptors in mediating pain and its multifarious expressions (Schaible et al., 2002; Neumann et al., 2008; see Paper I for more references). Intriguingly, prior to the work presented in this thesis, no human-based studies had been carried out to examine the role of CTs in allodynia.

4. CT-mediated allodynia: a précis of findings across four papers

In this thesis, detailed psychophysical observations of mechanical allodynia and its peripheral substrates – in particular the contribution of CT fibres to this effect – are presented as a series of four papers. Each of the papers pursues a specific question: 

Paper I. do CT fibres contribute to the generation of mechanical allodynia in hairy skin during acute muscle pain?; Paper II. is the peripheral substrate of allodynia in hairy and glabrous skin functionally analogous?; Paper III. is a ‘perceptual’ level of pain necessary to unmask the allodynic effect of CT-fibre activation, or can it be elicited by sub-perceptual events?; in addition, can CT-mediated allodynia be reproduced in a more persistent, or clinical, pain-state?; and finally Paper IV. can the expression of CT-mediated allodynia during deep somatic pain be extrapolated to a cutaneous pain-state? The unique features of each of the four papers are illustrated in Table 1. Whether these methodological variations are deemed subtle or profound, the essence of the observations remains unchanged. That is, a normally innocuous/pleasant tactile stimulus elicits allodynia during conditions of background nociceptive activity (perceptual or otherwise), and this effect is evidently mediated by low-threshold cutaneous mechanoreceptors with C fibres. In substantiation of this conclusion, the following points are worthy of note: i. vibration and brushing stimuli
are invariably and unequivocally innocuous under normal conditions, and have a demonstrated capacity to activate low-threshold mechanoreceptors – brushing, in particular, has been extensively used for activation of CT fibres; ii. in Papers I-III, a two-compartment model was adopted wherein tactile stimuli and pain-inducing manipulations were targeted at distinct anatomical compartments (skin and muscle), thereby circumventing altered responsiveness (sensitization) of cutaneous nociceptors; iii. vibration- and brush-evoked allodynia persisted following conduction block of the myelinated afferents by compression; and iv. allodynia was abolished during conduction block of cutaneous C fibres by injecting a local anaesthetic into the skin stimulated by vibration while the myelinated fibres were conducting or not. Relative to a punctate mechanical stimulus (vibration), a moving mechanical stimulus (brushing) excites a much larger area of skin, thereby making it unfeasible to selectively block the C fibres by an intradermal injection of local anaesthetic. Nonetheless, brushing stimuli were applied at speeds known to generate a particularly profound response in CT fibres, in addition to the persistence of brush-evoked allodynia following conduction block of myelinated fibres. In conclusion, these psychophysical observations provide the first human evidence of the role of CT fibres in pain processing. This finding contributes to our understanding of the pathophysiology underlying various ‘pain’ conditions, which may result in the development of novel drug targets/pain therapies to treat the actual source rather than the symptoms of pain.
Table 1. Methodological Variations Across Four Papers Aimed at Determining CT-functionality in Context of Pain

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<th>Participants</th>
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<th>Noxious stimulation</th>
<th>Spinal segments innervating tactile &amp; noxious stimulation sites</th>
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- ■: Indicates the use of that stimulus or skin type in the respective paper.
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References


PAPER I

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Allodynia mediated by C-tactile afferents in human hairy skin

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Non-technical summary What triggers a realignment of sensations, e.g. a stimulus that is perceived as non-painful in intact skin, but evokes pain in sunburned skin, is yet to be ascertained. This phenomenon is clinically termed allodynia. We show that gentle tactile stimulation (vibration and brushing) of the hairy skin can exacerbate the underlying muscle pain (allodynia) evoked by infusion of hypertonic saline into the tibialis anterior muscle. This effect is dependent upon a low-threshold, mechanosensitive class of nerve fibres in the hairy skin known as C-tactile (CT) fibres. Knowledge of the role of CT fibres in allodynia increases our understanding of the mechanisms that underlie sensory-perceptual abnormalities – a common manifestation of clinical-pain states and neurological disorders.

Abstract We recently showed a contribution of low-threshold cutaneous mechanoreceptors to vibration-evoked changes in the perception of muscle pain. Neutral-touch stimulation (vibration) of the hairy skin during underlying muscle pain evoked an overall increase in pain intensity, i.e. allodynia. This effect appeared to be dependent upon cutaneous afferents, as allodynia was abolished by intradermal anaesthesia. However, it remains unclear whether allodynia results from activation of a single class of cutaneous afferents or the convergence of inputs from multiple classes. Intriguingly, no existing human study has examined the contribution of C-tactile (CT) afferents to allodynia. Detailed psychophysical observations were made in 29 healthy subjects (18 males and 11 females). Sustained muscle pain was induced by infusing hypertonic saline (HS: 5%) into tibialis anterior muscle (TA). Sinusoidal vibration (200 Hz–200 μm) was applied to the hairy skin overlying TA. Pain ratings were recorded using a visual analogue scale (VAS). In order to evaluate the role of myelinated and unmyelinated cutaneous afferents in the expression of vibration-evoked allodynia, compression block of the sciatic nerve, and low-dose intradermal anaesthesia (Xylocaine 0.25%) were used, respectively. In addition, the modulation of muscle pain by gentle brushing (1.0 and 3.0 cm s⁻¹) – known to excite CT fibres – was examined. Brushing stimuli were applied to the hairy skin with all fibres intact and following the blockade of myelinated afferents. During tonic muscle pain (VAS 4–6), vibration evoked a significant and reproducible increase in muscle pain (allodynia) that persisted following compression of myelinated afferents. During compression block, the sense of vibration was abolished, but the vibration-evoked allodynia persisted. In contrast, selective anaesthesia of unmyelinated cutaneous afferents abolished the allodynia, whereas the percept of vibration remained unaffected. Furthermore, allodynia was preserved in the adjacent non-anaesthetized skin. Conformingly, gentle brushing produced allodynia (at both brushing speeds) that persisted during the blockade of myelinated afferents. Prior to the induction and following cessation of muscle pain, all subjects reported vibration and brushing as non-painful (VAS = 0). These results demonstrate that CT fibres in hairy skin mediate allodynia, and that CT-mediated inputs have a pluripotent central effect.

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Abbreviations CT, C-tactile; HS, hypertonic saline; TA, tibialis anterior muscle; VAS, visual analogue scale.
Introduction

Within the somatosensory system, a sensory relay is often described in terms of a ‘labelled line’ that links primary afferents with higher-order neurons in the primary somatosensory cortex via modality-specific spinal pathways. It is widely accepted that discriminative touch is mediated exclusively by large-diameter sensory fibres, whereas painful sensations are mediated by small-diameter fibres. Consistent with this view, selective microstimulation of a single large-diameter myelinated afferent in awake human subjects evokes a fundamental, innocuous (non-painful) sensation that has the quality of pressure, flutter or vibration according to the type of primary afferent excited (Ochoa & Torebjork, 1983; Vallbo et al., 1984; Macefield et al., 1990). Furthermore, the vibrotactile modalities are abolished in patients with large-fibre neuropathy (Olausson et al., 2002) and healthy subjects following blockade of large-diameter axons (Mackenzie et al., 1975; Dellon, 1980). By contrast, activation of small-diameter nociceptors in skin evokes sensations that are distinctly painful – sharp-stabbing for Group III fibres (fast pain) and burning for Group IV fibres (slow pain) – whereas activation of either fibre class in muscle produces sensations that are frequently described as dull and aching in quality and difficult to localize (Torebjork et al., 1984a, b; Ochoa & Torebjork, 1989). In addition to cutaneous nociceptors, which have high mechanical thresholds, there is another class of unmyelinated (C) fibre that has low mechanical thresholds. The existence of low-threshold unmyelinated afferents, termed C-mechanoreceptors, which respond to light touch of the skin, was documented long ago in the hairy skin of the cat and monkey (Zotterman, 1939; Maruhashi et al., 1952; Douglas & Ritchie, 1957; Bessou et al., 1971). Although some investigators had suggested that C low-threshold mechanoreceptors (CLTs) are vestigial (Kumazawa & Perl, 1977), recent studies have reported a class of unmyelinated fibres in the human hairy skin, known as C-tactile (CT) fibres, that responds to innocuous mechanical stimulation (Johansson et al., 1988; Nordin, 1990; Vallbo et al., 1993).

The response properties of CT fibres have been described using a limited range of stimuli – most notably slowly moving, low-force, mechanical stimuli such as finger stroking and soft brushing (Nordin, 1990; Vallbo et al., 1993, 1999; Loken et al., 2009). Likewise, our limited understanding of the contribution of CT fibres to perception warrants further exploration, as our current understanding of their role is based largely on observations in two subjects with large-fibre sensory neuropathy. In these patients the perceptual responses to light mechanical stimuli varied from trial to trial, but on occasion patients reported a faint sense of pleasantness. It is this latter observation, together with the results of neuroimaging studies that have demonstrated that CT-mediated inputs project onto the insular cortex, which has underpinned the proposition of a CT-mediated emotional touch system (Olausson et al., 2002; Cole et al., 2006; McGlone et al., 2007; Olausson et al., 2008). Intriguingly, in healthy subjects gentle brushing – known to elicit CT fibre responses – can evoke a neutral or even unpleasant sensation at the lowest brushing velocities (Loken et al., 2009), suggesting that gentle tactile stimulation can elicit opposing aspects of touch, i.e. predilection and aversion. A contribution of CT fibres to unpleasant touch has been suggested by recent work showing the activation of superficial dorsal horn neurons by gentle brushing of skin (Andrew, 2010; Craig, 2010). Similarly, these fibres have been implicated in touch hypersensitivity after injury in mice (Seal et al., 2009).

In a recent pilot study we found that innocuous tactile stimulation (vibration) of hairy skin intensified the underlying muscle pain (allodynia), and that this effect appeared to be dependent upon cutaneous mechanoreceptors as the allodynia was abolished by intradermal anaesthesia (Nagi et al., 2009). In previous studies, allodynia has often been attributed to the activation of large-diameter tactile afferents (Campbell et al., 1988; Treede & Cole, 1993; Wasner et al., 1999; Mailhöfner et al., 2003). Consistent with this view, touch-evoked allodynia has been abolished by compression or ischaemic blockade of large-diameter fibres (Gracely et al., 1992; Torebjork et al., 1992; Koltzenburg et al., 1994; Cervero & Laird, 1996). By contrast, other studies have argued for the involvement of small-diameter nociceptive afferents based on the persistence of allodynia following large-fibre blocks (Cline et al., 1989; Price et al., 1992). Moreover, allodynia can be abolished by anaesthesia of small-diameter presumed nociceptors in patients with ongoing pain (Arner et al., 1990; Gracely et al., 1992; Koltzenburg et al., 1994). Whether there is a contribution of CT fibres to allodynia remains untested.

The ambiguity in the literature about the contribution of different fibre classes to allodynia may be attributed in part to the use of a single-compartment model in which innocuous and noxious stimuli are applied to the same or adjacent regions of skin. Such an approach can lead to uncertainty as to whether any change in pain perception reflects peripheral sensitization of nociceptive fibres and/or an altered central convergence of innocuous and noxious inputs. In order to avoid this ambiguity in the present study we used a two-compartment model. Pain was induced in the tibialis anterior muscle (TA) by infusion of hypertonic saline and a neutral tactile stimulus – a low-amplitude (200 μm) vibration (200 Hz) – applied to the overlying skin. The muscle is physically separated from the skin by sheet-like fascia and each is supplied by separate vascular and nerve supplies (O’Rahilly & Muller, 1986; Berry et al., 1995; Salmons,
1995; Gibson et al. 2009). Within the hairy skin it is known that such low-amplitude vibratory stimuli are preferentially encoded by hair follicle afferents at low frequencies (~5 Hz to 100 Hz) and by Pacinian corpuscle receptors at high frequencies (~50 Hz to 1000 Hz: Merzenich & Harrington, 1969; Mahns et al. 2006). Although the response properties of CT fibres to vibratory stimulation remain untested, low-threshold mechanical sensitivity has been demonstrated using soft brushing (Vallbo et al. 1999; Olaussen et al. 2002; Loken et al. 2009). Furthermore, it has been shown that the perceptual outcome of gentle brushing corresponds best with the CT-fibre responses indicating the ‘preferential’ nature of the resulting stimulus (Loken et al. 2009). The use of innocuous tactile stimuli, i.e. vibration and brushing, coupled with differential nerve blocks, allowed us to disentangle the affective and sensory components of touch and thus quantify the perceptual response of CT fibres in healthy subjects.

Methods

Twenty-nine healthy human subjects (18 males and 11 females) aged 18–38 years, with no reported musculoskeletal disorders, took part in this study. Informed consent was obtained from each subject in writing. All experiments were approved by the UWS Human Research Ethics Committee and conformed to principles of the Declaration of Helsinki. In experimental Series I, we examined the effect of cutaneous vibration on muscle pain following compression of the myelinated afferents (sciatic nerve). A small amount of non-selective local anaesthetic (Xylocaine 2%) was injected intradermally in order to abolish the residual cutaneous input. In experimental Series II, we recorded the modulation of pain by vibration prior to the selective blockade (Xylocaine 0.25%) of unmyelinated cutaneous afferents, within the anaesthetized skin (C fibres blocked) and in the adjacent, non-anaesthetized skin with all fibres intact. In experimental Series III, we used gentle brushing over the hairy skin, with all fibres intact and following compression of myelinated afferents. Subjects sat comfortably on a chair with both legs supported horizontally for the vibration experiments or vertically for the brushing experiments. The anatomical boundaries of TA were identified by palpation during active inversion of the foot and dorsiflexion of the ankle joint (Gibson et al. 2006).

Hypertonic saline-induced muscle pain

A 23 G butterfly cannula was inserted through the skin into TA (~6 cm distal to the tibial tuberosity and ~2 cm lateral to the anterior border of tibia) and connected to an infusion pump (model 55–2226, Harvard Apparatus, Holliston, MA, USA) containing hypertonic saline (HS: 5%). The initial infusion rate was set at 200 μl min⁻¹ and once a peak level of pain had developed the infusion rate was adjusted (where needed) in order to maintain a constant pain rating of 4–6 on a visual analogue scale (VAS). The VAS was divided into 10 equal segments within a range of 0 (no pain) to 10 (worst pain). Beyond these initial adjustments, a stable baseline muscle pain was maintained throughout the duration of HS infusion, i.e. ~15 min, without further adjustments to the infusion rate. A stable baseline pain for at least 2 min was required prior to the concurrent application of tactile stimuli. Subjects reported pain by rotating a calibrated potentiometer, the signal from which was recorded continuously on a computer.

Cutaneous vibration

A circular Perspex (Plexiglas) probe with a rounded 4 mm diameter tip was placed in gentle contact with the skin overlying TA without compressing the underlying structures. Subjects were asked to ensure there was no discomfort around the site of contact (VAS = 0). The probe was positioned perpendicular to the skin surface at a point distal to the muscle belly and the cannula delivering the hypertonic saline, i.e. ~15 cm distal to the tibial tuberosity and ~1.5 cm lateral to the anterior border of tibia. The probe was attached to a feedback controlled sinusoidal mechanical stimulator (see Mahns et al. 2006). The frequency (200 Hz) and amplitude (200 μm) parameters of the stimulus were chosen as being unequivocally innocuous (Merzenich & Harrington, 1969; Mahns et al. 2006; Sahai et al. 2006) and devoid of any affective or emotional quale. The sensory neutrality of vibration was confirmed in each subject at the start of the experiment. Vibration was applied before, during and after the HS-induced muscle pain. During muscle pain, at least three consistent, consecutive vibration-evoked increases in muscle pain (allodynia) were required before progressing with the experiment. The period of vibration lasted 30 s and was repeated at 45 s intervals in order to provide sufficient time between trials and avoid desensitization of the activated fibre classes due to repeated stimulation (Iggo, 1960; Bessou et al. 1971). White noise was delivered through headphones to ensure that auditory cues associated with the mechanical stimulator were not evident to the subject (Merzenich & Harrington, 1969).

Series Ia. Compression of myelinated afferents

In 14 subjects, compression block of the sciatic nerve was used to evaluate the contribution of myelinated afferents
to the expression of allodynia. A metal bar was placed just distal to the ischial tuberosity to apply compression to the sciatic nerve. Commencement of the HS infusion was timed to coincide with the preferential blockade of large- and small-diameter, myelinated afferents (Laursen et al. 1999). In the intervening period (45 s) between consecutive vibration trains, selective sensory stimuli were applied to test the progression of nerve block. Myelinated blockade was confirmed by the loss of perception of vibration (20 and 200 Hz; 200 μm; 30 s duration), innocuous cold (~15°C, brass rod in contact with the skin for 5 s) and pinprick (applied to the skin using a sterile hypodermic needle) stimuli. The integrity of C-fibre inputs was confirmed by the preservation of warm sensibility (~40°C, brass rod in contact with the skin for 5 s: Mackenzie et al. 1975; Weerakkody et al. 2003). To verify the effectiveness of sciatic nerve compression, somatosensory sensibility was compared with skin regions on the medial aspect of the experimental leg, which is innervated by the femoral nerve (Berry et al. 1995), and the contralateral leg. When performing these tasks, subjects were shielded from visual and auditory cues.

Series IIb. Anaesthesia of residual cutaneous afferents

After recording vibration-evoked effects during the myelinated-fibre block, 0.2–0.4 ml of local anaesthetic (Xylocaine 2%) was injected intradermally into a 2–3 cm area surrounding the vibration probe. This small intradermal injection effectively blocked the residual inputs arising from skin without affecting the muscle nociceptors that mediate HS-induced pain (Arner et al. 1990; Mahns et al. 2006). The blockade of C afferents—the only class of fibres intact during compression of the myelinated axons—in the anaesthetized skin was verified by the abolition of warm sensibility. VAS responses to vibration were recorded within the anaesthetized skin (all fibres blocked) and in the adjacent non-anaesthetized skin (C afferents intact) within the innervation territory of the sciatic nerve to determine whether the pre-anaesthetic vibration-evoked response was preserved.

Series II. Anaesthesia of unmyelinated (C) afferents

This was carried out on a total of 10 subjects, including four subjects who had previously participated in experimental Series I and II. A paintbrush (0.7 cm thick and ~7.5 cm wide) of goat’s hair (3.0 cm long) was swept bi-directionally, perpendicular to the skin surface along the anterolateral aspect of leg. The stimulus brush was moved at speeds of 1.0 cm s⁻¹ or 3.0 cm s⁻¹ through a stroke of 10.0 cm plus a 1.0 cm turnaround at each end. The brushing motion was produced using a linear motor on a 3D Gantry system (Baldor Australia Pty Ltd, Seven Hills, NSW, Australia) that was under feedback of a PMAC motion controller (Delta Tau Data Systems, Inc., Chatsworth, CA, USA). These brushing speeds were chosen as CT fibres respond particularly well to these stimuli, which are perceived as pleasant (Loken et al. 2009). The non-noxious nature of the brushing was confirmed in each subject at the start of the experiment. Brushing stimuli were delivered before, during and after the HS-induced muscle pain—with all fibre classes intact and following blockade of myelinated afferents. During muscle pain, VAS responses to brushing were recorded for each of the two brushing speeds. Akin to vibratory stimulation, the period of brushing lasted 30 s and was repeated at 45 s intervals. White noise was delivered through headphones to ensure that auditory cues associated with the brushing system were not provided (Merzenich & Harrington, 1969).

Statistical analysis

Data are presented as means ± standard error of the mean (±SEM). In each individual, the responses to vibration or brushing were expressed as a percentage of the HS-induced muscle pain (Base) observed immediately preceding the superimposition of tactile stimuli (Figs 2–5). Each touch-evoked change in muscle pain was treated as
an independent, sequential event. Significant changes were detected using one-way analysis of variance (ANOVA; Zar, 1984). Where significant differences were indicated ($P < 0.05$), individual groups were compared using a Newman–Keuls multiple comparison test. All statistical comparisons were made using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

**Results**

**Pain induced by intramuscular infusion of hypertonic saline and concurrent tactile stimulation**

In 22 of the 25 subjects where hypertonic saline was infused, a constant level of pain within a VAS range of 4–6 was reported. On average, it took about 3–5 min to attain a stable baseline pain that persisted for the duration of HS infusion. Data from the remaining three subjects were discarded because a constant background level of pain could not be obtained. Prior to the induction of muscle pain, all subjects reported that cutaneous vibration and brushing were not painful (VAS = 0) when applied to the hairy skin. Infusion of hypertonic saline into the TA evoked sensations of a dull ache that radiated distally from the injection site and in 12 subjects extended (referred) beyond the ankle. In the presence of sustained muscle pain, cutaneous vibration and gentle brushing evoked an increase in muscle pain (allodynia). This allodynia was reproducible over time and disappeared upon cessation of the underlying muscle pain. At this point, the vibratory/brushing stimuli were once again described as being non-painful (VAS = 0). Raw data from one subject and mean data of two consecutive vibration-evoked responses are shown in Fig. 1. Gentle brushing evoked a cognate expression of allodynia.

**Series Ia. Vibration-evoked allodynia persists during compression of myelinated afferents**

Compression block of the sciatic nerve was used to test whether allodynia can be evoked in the absence of myelinated afferents. In 4 of the 14 subjects, effective blockade of myelinated afferents could not be achieved, presumably because the metal bar had been placed incorrectly. Hence, hypertonic saline was not used in these subjects. During compression, the time taken to fully block the perceptual responses associated with myelinated afferents varied from ~30 to 45 min across subjects. The blockade of large- and small-diameter, myelinated afferents was confirmed in each subject by the loss of perception of vibratory, innocuous cold and pinprick stimuli. The integrity of C-fibre inputs was confirmed by the preservation of warm sensibility. The mean data (±SEM) demonstrated that muscle pain increased significantly during vibration (allodynia), was reproducible over time and ebbed before the onset of subsequent periods of vibration (base: 100%; vibration: $119.7 \pm 3.8\%$, $119.1 \pm 3.5\%$; $P < 0.001$; $n = 10$). Apart from the vibration-evoked allodynia, there was no percept of vibration during the compression block. No changes in the intensity of the underlying muscle pain (base) or the magnitude of vibration-evoked allodynia were observed ($P > 0.05$) in the responses to consecutive vibration trains. Mean data (% control) of successive vibration-evoked responses in duplicate sets are shown in Fig. 2.

**Series Ib. Vibration-evoked allodynia was abolished by anaesthesia of residual cutaneous afferents**

Following compression block, intradermal anaesthetic was used to abolish all residual inputs from the skin.

![Figure 1](image-url). *Effect of vibration before, during and after hypertonic saline-induced muscle pain* VAS recordings over time of a typical subject (left) and the mean data of successive vibration-evoked responses (±SEM; $n = 7$; grey: allodynia; unshaded: baseline muscle pain) are shown. Cutaneous vibration (200 Hz–200 µm) evoked a reproducible increase in muscle pain throughout the sustained level of background pain (VAS 4–6), as well as during the decay phase following cessation of hypertonic saline (HS) infusion. Vibration-evoked allodynia ebbed before the onset of subsequent vibration trains. Prior to the initiation and on termination of HS-induced muscle pain, vibration was reported as non-painful (VAS = 0).
The effectiveness of the block was verified by the abolition of warm sensibility. The baseline pain remained unaffected, confirming that muscle nociceptor afferents were not affected. Following cutaneous anaesthesia, vibration had no effect on the underlying muscle pain, i.e. the allodynia was abolished (base: 100%; vibration: 100.2 ± 0.2%, 100.0 ± 0.0%; P > 0.05; n = 10). In those subjects in whom vibration was applied in the adjacent non-anaesthetized skin (C fibres intact) – within the innervation territory of sciatic afferents – vibration evoked a significant increase (allodynia) in the baseline HS-pain, i.e. allodynia was preserved (base: 100%; vibration: 125.1 ± 5.2%, 129.9 ± 2.0%; P < 0.001; n = 7). Thus, allodynia was evoked regardless of the blockade of myelinated afferents, but was abolished by the inactivation of unmyelinated cutaneous afferents. Conversely, in the adjacent (non-anaesthetized) skin with intact C afferents, allodynia was preserved (ANOVA: F = 21.12, P < 0.0001; Fig. 2).

Series II. Vibration-evoked allodynia mediated by C-tactile afferents

Low-dose intradermal anaesthetic (Xylocaine 0.25%) was used to test whether allodynia persists following the inactivation of unmyelinated fibres. Preferential blockade of C afferents was confirmed by the abolition of warm sensibility and a preserved appreciation of vibration and cold stimuli. The use of intradermal anaesthetic had no perceptible effect on the intensity of baseline muscle pain. Prior to intradermal anaesthesia (all fibres intact), the mean data (±SEM) demonstrate that muscle pain significantly increased during vibration (base: 100%; vibration: 115.0 ± 3.6%, 118.9 ± 4.8%; P < 0.01; n = 7). No changes in the intensity of muscle pain (base) or the magnitude of vibration-evoked allodynia were observed (P > 0.05) in successive vibration trains. Moreover, the vibration-evoked allodynia disappeared before the onset of subsequent vibration trains. Following the blockade of unmyelinated cutaneous afferents, vibration had no effect on the underlying muscle pain, i.e. the allodynia was abolished (base: 100%; vibration: 103.3 ± 3.2%, 100.0 ± 4.9%; P > 0.05; n = 6). In one of the seven subjects, intradermal anaesthetic was not administered due to the emergence of an unstable baseline. Furthermore, in two of the remaining six subjects, an additional injection of low-dose anaesthetic (0.2 ml) was required in order to abolish the warm sensibility. In contrast to the blockade of myelinated afferents where allodynia was preserved but the percept of vibration was abolished, the blockade of unmyelinated cutaneous afferents abolished allodynia while the sense of vibration was unaffected. In the adjacent non-anaesthetized skin, vibration evoked a significant increase in the underlying muscle pain, i.e. the allodynia was preserved (base: 100%; vibration: 122.9 ± 5.8%, 119.0 ± 4.7%; P < 0.001; n = 6). Thus, allodynia was evoked prior to anaesthesia of unmyelinated afferents or in the adjacent skin with intact cutaneous afferents, whereas allodynia was abolished within the anaesthetized skin (ANOVA: F = 8.399, P < 0.0001). Mean data (% control) of sequent vibration-evoked responses in duplicate sets are illustrated in Fig. 3.

Series III. Brush-evoked allodynia mediated by C-tactile afferents

Gentle brushing was used to confirm the role of CT fibres in allodynia, given the documented capacity of brushing stimuli for generating a particularly profound response in CT fibres (Loken et al. 2009). Prior to the induction of muscle pain, all subjects described brushing as non-painful (VAS = 0) and devoid of negative affective qualities. By contrast, during muscle pain the mean data (±SEM) demonstrate that brushing at 1 cm s\(^{-1}\) and 3 cm s\(^{-1}\) evoked a significant and reproducible increase in the pain intensity that abated before the onset of subsequent periods of brushing (base: 100%; brushing at 1.0 cm s\(^{-1}\): 127.0 ± 4.1, 126.8 ± 6.0; brushing at 3.0 cm s\(^{-1}\): 125.2 ± 5.2, 126.7 ± 5.5; ANOVA: F = 14.52, P < 0.0001; n = 9). This effect was not dependent upon intact myelinated fibre inputs, as brush-evoked allodynia was preserved, or amplified, following compression...
we have shown that innocuous cutaneous vibration can increase the intensity of underlying muscle pain, induced by intramuscular infusion of hypertonic saline, and that this effect (i) persists during compression blockade of myelinated fibres but (ii) is abolished by selective anaesthesia of unmyelinated cutaneous afferents. Thus, vibration-evoked allodynia is evidently dependent upon intact C fibre inputs from the skin, and that these C-fibres have a low mechanical threshold (they responded to 200 µm vibration). The abolition of allodynia was not associated with a decline in the muscle pain or a generalised decline in the capacity of vibration to evoke allodynia, as the vibration-evoked increases in pain were preserved in the adjacent non-anaesthetized skin. Vibration was described as non-painful by all subjects prior to the induction, and following cessation, of muscle pain. Our observations clearly implicate the mechanically sensitive C-tactile (CT) fibres in mediating this vibration-evoked allodynia. In contrast to earlier work, our psychophysical data indicate that the mechanical sensitivity of CT fibres need not be limited to slowly moving stimuli, as allodynia was evoked by vibration following blockade of myelinated afferents.

Although the responsiveness of CT fibres to vibratory stimuli remains untested, recent studies have revealed a broader range of response properties, such as delayed acceleration (Vallbo et al. 1999), that may account for the vibrotactile responsiveness shown in this study. To our knowledge, this is the first study that has demonstrated a distinct function attributable to C-tactile fibres in human subjects with a full complement of sensory fibres. Much of the recent work has focused on CT fibres in the context of an emotional touch system, largely based on the variable reports of neutral or pleasant responses to gentle brushing in two patients with loss of large-fibre function (Olausson et al. 2002, 2008; Cole et al. 2006; McGlone et al. 2007). However, brush stroking is not a CT-specific stimulus and is known to also activate large-diameter tactile afferents (Krämer et al. 2007; Loken et al. 2009; Andrew, 2010). Using the same data presented by Loken et al. (2009) an alternative explanation can be advanced, namely that C-fibre and large-diameter afferents are activated in parallel during brush stroking, with a sense of pleasantness emerging when large-diameter responsiveness exceeds that of C-fibres. Conversely, in
their study neutral or unpleasant (negative pleasant) scores were reported at the lowest brushing velocities, where CT responsiveness may well exceed the activation of large-diameter tactile afferents, suggesting that the role C-tactile fibres need not be confined to pleasant touch. Likewise, it is intriguing that the reported absence of CT fibres in glabrous skin does not preclude our capacity to perceive the pleasantness of affective touch stimuli such as brush-stroking or velvet-fabric (Rolls et al. 2003; Krämer et al. 2007). In our study, brushing stimulation – at reportedly pleasant speeds – evoked allodynia during muscle pain. Thus, it is the concurrent activation of muscle nociceptors during hypertonic saline infusion and cutaneous mechanoreceptors during brushing (and vibratory) stimulation that leads to the allodynia. The use of differential nerve blocks to avoid the co-activation of multiple fibre classes during tactile stimulation – an ambiguity that has plagued earlier studies – confirms the role of CT fibres in mediating allodynia. Hence, it is the complement of active sensory fibres, rather than the activation of a single class of afferents, which determines the perceptual outcome of activating CT fibres. Our observations are indeed consistent with a role of CT fibres in emotional touch, in particular the crossover between neutral-touch and painful-touch (allodynia). Furthermore, the abolition of vibratory sense during myelinated-compression block argues against the role of CT fibres in discriminative touch. Whether they can play a more circumscribed role in the detection of punctuate stimuli warrants further investigation, given the report that patients with large-fibre sensory neuropathy are able to detect weak monofilaments on the hairy skin (Cole et al. 2006).

The contribution of large-diameter tactile afferents and small-diameter nociceptors to allodynia has been extensively studied (Campbell et al. 1988; Cline et al. 1989; Price et al. 1992; Torebjork et al. 1992; Koltzenburg et al. 1994; Maihöfer et al. 2003). However, the role of CT fibres in mediating allodynia is a novel observation. The contribution of C-tactile afferents may have been obscured due to the use a single-compartment model, in which innocuous and noxious stimuli are applied to the same or adjacent regions of skin. As noted in the Introduction, such an approach can lead to uncertainty as to whether any change in pain perception reflects peripheral sensitization of nociceptive fibres or an altered central convergence of tactile and nociceptive inputs. In this study such ambiguities were avoided by adopting a two-compartment approach; however it remains to be tested whether CT-mediated allodynia is limited to heterologous structures such as skin and muscle or can be produced by inputs arising from cutaneous nociceptors and CT fibres.

Studies in experimental animals have shown that CT input can activate ‘modality-ambiguous’ or wide dynamic range lamina I neurons (Keller et al. 2007; Andrew, 2010). Furthermore, nociceptive lamina I neurons display increased responsiveness during CT input following peripheral nerve injury (Keller et al. 2007). These observations provide a possible pathway by which mechanoreceptive impulses in CT fibres converge with inputs from muscle nociceptors and transmit to the higher centres. Although musculosomatic convergence has been demonstrated in experimental animals (Foreman et al. 1979), this is the first study to unravel the role of CT fibres in altered central integration of cross-modality inputs thereby eliciting allodynia. CT-related processing is often described as occurring below a conscious level, which is consistent with a lack of distinct quality of sensation (Olausson et al. 2010). Whether activation of CT afferents is associated with a unique set of qualia needs to be systematically studied using affective- (positive and negative) and neutral-touch stimuli in protocols that do not limit the resulting sensation to the persistence of background pain.

What triggers a crossover between pain and pleasure during affective- or neutral-touch processing is yet to be ascertained. Neuroimaging studies have shown differential

![Figure 5. Allodynia evoked by gentle brushing in absence of myelinated afferents](image)

Mean brush-evoked responses (±SEM; n = 5; grey) were expressed as a percentage of the baseline muscle pain (unshaded). Gentle brushing at speeds of 1 cm s⁻¹ and 3 cm s⁻¹ evoked a significant increase in HS-induced muscle pain (allodynia) during compression of myelinated afferents. The expression of allodynia was comparable between the two brushing speeds. Note that scaling is different in this figure. **P < 0.01; ***P < 0.001.
representation of pleasant and painful tactile stimuli in certain areas of the brain involved in emotional processing (insula, orbitofrontal and anterior cingulate cortices: Olausson et al. 2002; Rolls et al. 2003). However, cortical activation evoked by a neutral tactile stimulus predominantly activates the discriminative-cognitive areas, the primary and secondary somatosensory cortices. It has been argued that the tactile allodynia experienced following administration of sumatriptan may be explained by an induced deficit in affective (pleasant) processing (Krämer et al. 2007). In contrast, our results strongly indicate that CT activation can mediate a crossover to the affective component of touch, i.e. neutral to painful.

The qualia of touch may have evolved mainly in a social context to create a useful construct of the world, e.g. to predict whether the intent behind another's action was benign or sinister; synthesized with the sense of 'self', these inputs subserve reflective self-awareness that characterizes humans as immensely social creatures (Ramachandran, 2004). Individuals with autism suffer from sensory-perceptual abnormalities such as hypersensitivity or defensiveness to touch stimuli (e.g. vibration), which in part at least contributes to social withdrawal (Baranek, 1999; Cascio et al. 2008). These observations in individuals with autism are consistent with our experimental results in healthy subjects, suggesting a role of CT afferents in behavioural responses such as sensory-avoiding. Clearly, the contribution of these seemingly primitive tactile fibres to the emotional touch system has immense implications for understanding self-regulating mechanisms that at least in part determine social behaviours.

References


Author contributions
All experiments were performed at the School of Medicine, University of Western Sydney. The contribution of each author to this study is as follows. Conception and design, or analysis and interpretation of data: all authors contributed; drafting the article or revising it critically for important intellectual content: all authors contributed; final approval of the version to be published: all authors approved.

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PAPER II

Modified version presented in Exp Br Res format
MECHANICAL ALLODYinia IN GLABROUS SKIN MEDIATED BY LOW-THRESHOLD CUTANEOUS MECHANORECEPTORS WITH UNMYELINATED FIBRES

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Abstract

We recently showed that C-tactile fibres (CTs) in human hairy skin (anterior leg) mediate crossover between innocuous-touch and noxious-touch, i.e. mechanical allodynia. Although there is no evidence for existence of a phenotypically identical class of CTs in human glabrous skin, the ‘qualia’ of affective stimuli are comparable across skin types. In 42 healthy subjects, muscle pain was induced by infusing hypertonic saline (5%) into flexor carpi ulnaris muscle. Concurrently, sinusoidal vibration (200 Hz-200 µm) was applied to glabrous skin of little finger. The neural substrate of allodynia was determined by employing conduction blocks of myelinated (ulnar nerve compression) and unmyelinated (low-dose intradermal anaesthesia) fibres. In order to compare the expression of allodynia across spinal segments and skin types, vibration was also applied to glabrous skin of index finger and hairy skin of dorsal forearm. In addition, high-precision brushing stimuli were applied at speeds of 1.0 and 3.0 cm s\(^{-1}\) to digital glabrous skin with absent myelinated fibres. During muscle pain, vibration caused a significant and reproducible increase in pain (allodynia). This effect persisted during blockade of myelinated fibres, but was abolished by inactivation of unmyelinated cutaneous fibres. The vibration-evoked effects were found to be comparable across spinal segments and skin types. Furthermore, brushing produced a cognate expression of C fibre-mediated allodynia. Prior to induction and upon cessation of muscle pain, vibration and brushing were reported as non-painful. Based on these results, we postulate that a functionally equivalent class to the CTs (hairy skin) mediates allodynia in human glabrous skin.

Keywords: C fibre; muscle pain; allodynia; glabrous skin; hairy skin; hypertonic saline; vibration; intradermal anaesthesia
Introduction

In everyday life, we can readily differentiate between innocuous (tactile) and noxious (painful) stimuli. However, the point that determines the transition between tactile and pain sensations often changes in pathological conditions, such that an otherwise non-painful tactile stimulus is perceived as painful. This switch in modality is characteristic of *mechanical allodynia*, which is often a therapeutically intractable feature of ‘pain’ conditions. The neural mechanisms underlying this phenomenon have been extensively studied in hairy skin, particularly in the context of cutaneous pain, although the bulk of the observations remain limited to the activity of large myelinated mechanoreceptors. There has been minimal appreciation of C mechanoreceptors over the years, which may be attributable to doubts over their existence in humans (absence of evidence from initial microneurography studies), and a tendency to question their functional relevance especially when contrasted to the well-defined role of their myelinated counterparts. However, in recent microneurography work, it has been reported that C low-threshold mechanoreceptors (CLTMRs), dubbed C-tactile fibres (CTs), are encountered almost as frequently as the myelinated fibres in hairy skin (Löken et al. 2009; Vallbo et al. 1999). Furthermore, in a large-fibre deafferented patient, gentle brushing of the hairy skin was described as a vague and diffuse sense of pressure that, when perceived, was of a positive-affective or pleasant quality – an effect ascribed to CTs (Olausson et al. 2002). Moreover, we recently showed that, in presence of acute muscle pain, CTs in the hairy skin mediate a crossover between neutral/positive touch and negative affective or painful touch, i.e. allodynia (Nagi et al. 2011). However, in the glabrous skin, while the existence of a phenotypically identical class of CTs remains to be established, the question as to whether this skin type is innervated by a functional...
homolog of CTs, much like the hair follicle afferents in hairy skin and Meissner corpuscles in glabrous skin, has not been examined as yet. Nonetheless, it is a recurrent observation in the literature that the affective ratings of gentle tactile stimuli are comparable across hairy and glabrous skin, although with an undefined neural substrate in the latter (Löken et al. 2011; McGlone et al. 2012).

Contrary to the hairy skin, the paucity of information on the peripheral neural mechanisms underlying allodynia in glabrous skin is noteworthy as an injury-evoked response in one skin type may be quite different to the other (Rendell et al. 2002; Boada et al. 2010). Moreover, many behavioural models of neuropathic pain interpret avoidance responses following stimulation of the glabrous skin (in subprimates) as a measure of injury-induced nociceptive activity (Decosterd and Woolf 2000; Zahn et al. 2002; Ririe et al. 2003) while hairy skin is the primary region of interest for most surgical incisions and/or accidental traumas (Andrews and Fitzgerald 2002; Duarte et al. 2005).

Multiple functional classes of low-threshold mechanoreceptors innervating the human glabrous skin have been categorized (Johansson 1978; Vallbo et al. 1984). Amongst them are the Meissner corpuscles (MCs) which are innervated by large myelinated afferents that encode vibrotactile stimuli (Talbot et al. 1968). In addition, MCs are innervated by C fibres that have been suggested to play a role in nociception (Johansson et al. 1999; Paré et al. 2001). Furthermore, other studies in glabrous skin have identified C-fibre terminals that do not express the peptidergic nociceptor markers (e.g. Zylka et al. 2005). It remains to be ascertained whether the ‘absence of evidence’ truly represents an ‘evidence of (CT-fibre) absence’ in the glabrous skin,
or can it be attributed to the following: differences across skin types in terms of receptor characteristics and biophysical properties (Boada et al. 2010; Montagna and Parakkal 1974); and a reduction in innervation density along a proximal-to-distal gradient (Kumazawa and Perl 1977; Li et al. 2011; Liu et al. 2007) combined with the increased likelihood of pronounced masking effect of sympathetic efferent activity in the distal regions (Löken et al. 2007). Furthermore, investigations using molecular/genetic tools (in mice) remain inconclusive: on the one hand tyrosine hydroxylase-labelled (TH-positive) presumed CLTMRs were not found in the dorsal root ganglion (DRG) neurons that innervate glabrous region, but on the other hand more than three-fourths of the TH-positive neurons expressed the vesicular glutamate transporter VGLUT3, which is found in a subset of DRG cells that innervate the glabrous skin (Li et al. 2011; Seal et al. 2009). In humans, our current understanding of CLTMRs is based on limited observations in two patients with sensory neuronopathy syndrome (loss of large-fibre function). In contrast to the hairy skin, wherein these patients reported a faint sense of touch during gentle tactile stimulation (presumably CT-mediated), no percept was evoked in the glabrous skin. However, whether these patients had a full complement of unmyelinated fibres is questionable, given that the warmth thresholds (typically C-fibre mediated) were outside the normal range of detection (Cole et al. 2006; Olausson et al. 2008).

In this study, akin to our earlier observations in hairy skin (Nagi et al. 2011), a two-compartment model was adopted: pain was induced in the flexor carpi ulnaris muscle (FCU) by infusion of hypertonic saline and innocuous tactile stimuli (vibration and brushing) were applied to the digital glabrous skin. Such an approach allowed us to avoid sensitization of nociceptive nerve endings (Raja et al. 1988) and to quantify the
affective/sensory contributions of mechanosensitive units in glabrous skin to ongoing muscle pain – with and without differential nerve blocks. Furthermore, in order to gauge the variability in responses to pain, the expression of allodynia was examined across spinal segments and skin types.

Some of the results have been published in abstract form (Nagi and Mahns 2010).

**Methods**

Forty-two healthy human subjects (27 males and 15 females, aged 18-40 years) with no reported musculoskeletal disorders participated in this study. All subjects were naive about the objectives of the experiments. Informed consent, in writing, was obtained from each subject. All experiments were approved by the UWS Human Research Ethics Committee (approval no.: H9190) and complied with the principles of the Declaration of Helsinki. This study had four objectives: **Series 1 and 2a.** to identify the peripheral substrate of mechanical allodynia in glabrous skin by examining the interactions between pain and vibration prior to and following nerve conduction blocks; **Series 2b.** to determine whether the peripheral substrate of allodynia in glabrous skin is a functional homolog of CTs (hairy skin) by applying brushing to digital glabrous skin with absent myelinated fibres. **Series 2c.** to investigate whether the production of allodynia is limited to inputs arising from the same spinal segment, e.g. ulnar nerve/C8 (FCU and little finger), or can allodynia elicit across spinal segments, i.e. FCU and skin regions innervated by the median nerve (C7, index finger); **Series 2d.** to compare the expression of allodynia between hairy and glabrous skin - in the former, low-threshold C fibres have been shown to
exist (Vallbo et al. 1999) and contribute to allodynia (Nagi et al. 2011) whereas, in the latter, their existence is yet to be established.

Each experimental series was conducted subsequently, not concurrently. Subjects sat with their hand in a supinated (palm-anterior) position on a model hand affixed to the bench top. The little and index fingers were secured to the base by gentle taping them at the level of middle phalanges or by using a putty-like synthetic adhesive. The anatomical boundaries of FCU were identified by palpation during active flexion and adduction of the wrist joint (Drake et al. 2005).

Muscle pain
In 39 subjects, muscle pain was induced by inserting a 23G butterfly cannula through the skin into FCU (~12 cm distal to elbow). The cannula was connected to an infusion pump (model 55-2226, Harvard Apparatus, Holliston, MA, USA) containing hypertonic saline (HS: 5%). FCU is the most medial of the muscles in the superficial layer of wrist flexors and is innervated by the ulnar nerve (mainly C8: Drake et al. 2005). The HS-infusion into FCU was commenced at 50 µl min\(^{-1}\) and where needed the infusion rate was adjusted in order to maintain a constant pain rating for the duration of the infusion, i.e. 15-20 min. The pain intensity was measured on a visual analog scale (VAS) that was divided into ten equal segments within a range of 0 (no pain) to 10 (worst pain). Subjects were instructed to report pain by rotating a calibrated potentiometer, the signal from which was recorded continuously on a computer. A stable baseline pain for at least 2 min was a prerequisite for concurrent application of tactile stimuli.
Cutaneous vibration

A circular Perspex (Plexiglas) probe with a rounded 4 mm diameter tip was positioned perpendicular to the skin surface without compressing the underlying structures. Subjects were inquired to ascertain the absence of any discomfort at the site of contact. The probe was attached to a feedback controlled sinusoidal mechanical stimulator (Mahns et al. 2006). Particular frequency (200 Hz) and amplitude (200 µm) parameters were adopted in order to generate a perceptible, innocuous stimulus with a demonstrated capacity to activate large myelinated mechanosensitive fibres and, based on our earlier work in hairy skin, apparently unmyelinated mechanoreceptive fibres as well (Talbot et al. 1968; Mountcastle et al. 1972; Mahns et al. 2006; Nagi et al. 2011).

Muscle pain and concurrent tactile stimulation

In 17 subjects, vibration was applied to the distal palmar pad of little finger prior to the initiation of HS-infusion, during muscle pain and following termination of HS-infusion (once the pain dissipated) while all fibres were intact. During muscle pain, at least two successive vibration-evoked increases in muscle pain were required before progressing with the experiment (Nagi et al. 2011). The period of vibration lasted 30 s and was repeated at 45 s intervals. This allowed sufficient time between trials for thermal testing during conduction blocks (see following text) and prevented desensitization of the mechanoreceptive fibre classes due to repeated stimulation (Iggo 1960). Brown noise was delivered through headphones to eliminate any auditory cues associated with the mechanical stimulator.
Series 1. Touch-Pain interactions during blockade of unmyelinated fibres

In 11 subjects of the 17 subjects in whom pain and vibration interactions were examined, ~0.2 ml of local anaesthetic (Xylocaine 0.25%) was injected into the distal palmar pad of little finger (vibration site), thereby producing a preferential blockade of C fibres in a ~1 cm (in diameter) region (Torebjörk and Hallin 1973; Mackenzie et al. 1975). The inactivation of C fibres within the anaesthetized region was confirmed by the abolition of warm sensibility (~40°C, brass rod in contact with the skin for 5 s). The intactness of myelinated fibres was confirmed by a preserved appreciation of vibration (20 and 200 Hz; 200 µm; 30 s) and innocuous cold (~15°C, brass rod in contact with the skin for 5 s) stimuli. Thermal stimuli were applied in the intervening period of 45 s between consecutive vibration trains (Nagi et al. 2011). After confirmation of the C-fibre conduction block, vibration was reapplied to the anaesthetized skin (C fibres blocked) and the adjacent non-anaesthetized skin (proximal palmar pad of little finger) with all fibres intact.

Series 2. Touch-Pain interactions during blockade of myelinated fibres

In a subsequent series of experiments involving 23 subjects, including 5 who had previously participated in experimental Series 1, compression block of the ulnar nerve was attempted in order to knock out the myelinated fibres. However, the block failed to take effect in 3 subjects, hence they were excluded (data not shown). A small metal slab was placed posteriorly just proximal to the medial epicondyle of humerus to apply compression to the ulnar nerve. Infusion of HS into FCU was timed to coincide with the preferential blockade of large- and small-diameter, myelinated fibres (Laursen et al. 1999), which was confirmed by the loss of perceptiveness to vibration (20 and 200 Hz; 200 µm; 30 s duration) and innocuous
cold (~15°C, brass rod in contact with the skin for 5 s) stimuli that were applied to the glabrous skin of little finger (Torebjörk and Hallin 1973; Mackenzie et al. 1975; Hallin and Torebjörk 1976). The integrity of cutaneous C fibres was confirmed by the persistence of warm sensation (~40°C, brass rod in contact with the skin for 5 s). Furthermore, the progression of the compression block was verified by comparing the somatosensory sensibility in the ulnar territory with the skin overlying the index finger, which is innervated by the median nerve (Drake et al. 2005), and the contralateral little finger. Subjects were shielded from visual and auditory cues while performing these tests.

**Series 2a. Vibration applied to digital glabrous skin with absent myelinated fibres**

The effect of vibration, applied to the distal palmar pad of little finger (only C fibres intact), on muscle pain was examined in 15 subjects. This was followed by an injection of ~0.2 ml of low dose anaesthetic (Xylocaine 0.25%) into the skin stimulated by vibration. The local anaesthetic was aimed at blocking cutaneous C fibres – the only class of fibres intact during conduction block of the myelinated axons (Nagi et al. 2011). The abolition of the residual cutaneous input from the vibration site was verified by the loss of warm sensibility. VAS responses to vibration were recorded within the anaesthetized skin and in the adjacent non-anaesthetized skin of proximal palmar pad of little finger where C fibres remained intact.

**Series 2b. Gentle brushing of digital glabrous skin with absent myelinated fibres**

In 5 subjects (3 from **Series 2a** plus 2 naive participants), gentle brushing was used in place of vibration following the blockade of myelinated fibres. Given that a
moving mechanical stimulus of this sort has been extensively used for the activation of CTs, it allowed us to investigate whether the interactions between touch and pain in glabrous skin are mediated by a functionally equivalent neural substrate. The brushing stimuli were applied using a robotic device known as Rotary Tactile Stimulator (RTS; used extensively by Håkan Olausson and Francis McGlone to study CT-mediated pleasant touch; for details see Löken et al. 2009; Löken et al. 2011). A soft makeup brush (width of brush: 1 cm; length of bristles: ~1.5 cm) was swept bidirectionally along a proximo-distal/disto-proximal axis, perpendicular to the skin surface in a rotary fashion. The stimulus brush was moved at speeds of 1.0 or 3.0 cm s\(^{-1}\), and calibrated normal force of 0.4 N, over the palmar aspect of little finger. Akin to vibratory stimulation, the brush strokes were applied for 30 s with an inter-stimulus interval of 45 s. The stimulus parameters were chosen in conformity with our previous observations on CT-mediated allodynia in hairy skin (Nagi et al. 2011), in addition to a particularly pronounced neural discharge displayed by CTs at these brushing speeds (Löken et al. 2009).

**Series 2c. Vibration applied across spinal segments**

In 13 subjects, all of whom participated in Series 2a, vibration was applied to the distal palmar pad of index finger, in addition to the little finger. This was aimed at exploring whether the modulation of deep somatic pain by skin stimulation is dependent upon an overlap in spinal projections from skin and muscle or can allodynia extend to adjacent spinal segments. The glabrous skin of index finger is innervated by the median nerve (C7), whereas the FCU is innervated by the ulnar nerve (predominantly C8: Drake et al. 2005).
Series 2d. Vibration applied across skin types

In 9 subjects (6 from Series 2a plus 3 naive participants), vibration was also applied to the hairy skin of dorsal forearm overlying the extensor digitorum muscle in order to compare the expression of allodynia across glabrous and hairy skin, given the known differences in skin mechanics/tissue compliance and receptor characteristics across the two skin types (Talbot et al. 1968; Merzenich and Harrington 1969; Boada et al. 2010; Moore 1970; Slugg et al. 2000).

The vibration-evoked effects across spinal segments and skin types were measured in continuity of a conduction block of myelinated fibres in the ulnar territory while all nerve fibres in the index finger and the hairy skin (dorsal forearm) were intact. This allowed us to compare the expression of allodynia in a skin region with only C fibres intact with the vibration-evoked effects in other regions with a full complement of sensory fibres. In order to avoid any visual cues, two identical mechanical stimulators were in contact with the skin overlying little finger, index finger or dorsal forearm at all times for this part of the study. At any given time, only one stimulator was used to apply vibration. The order of testing across spinal segments and skin types was randomized amongst subjects in order to eliminate any order effects or response learning (Löken et al. 2011).

Statistical analysis

Each touch-evoked response was expressed as a percentage of baseline muscle pain (Base) reported just prior to tactile stimulation (Figs. 2-3) and treated as an independent, sequential event. Significant touch-evoked changes in muscle pain were detected using one-way analysis of variance (ANOVA: Zar 1984). Where significant
differences were found (P < 0.05), individual groups were compared using a
Newman-Keuls multiple comparison test. Prism 5 (Graph Pad Software, Inc., La
Jolla, CA, USA) was used for all statistical comparisons.

**Results**

In 32 of the 39 subjects where hypertonic saline was infused, a stable baseline pain
was attained within ~5 min that persisted for the duration of HS-infusion. Data from
4 subjects were discarded because a steady background pain could not be obtained.
In another 3 subjects, the experiments were discontinued due to a presumed
vasovagal reaction to actual or anticipated pain (van Lieshout et al. 1991). HS was
not used in those subjects (n = 3) where the effectiveness of the compression block
could not be confirmed. Prior to the induction of muscle pain, all subjects reported
that cutaneous vibration was non-painful (VAS = 0). Infusion of hypertonic saline
into the FCU evoked sensations of a dull ache that radiated distally from the injection
site and in 11 subjects extended (referred) beyond the wrist. During muscle pain,
cutaneous vibration applied to the little finger evoked an increase in the pain
intensity (Base: 3.6 ± 0.5, 3.4 ± 0.5; Vibration: 4.7 ± 0.5, 4.6 ± 0.5; n = 17). This
effect was reproducible and transitory, as it dissipated during the inter-vibration
interval. Furthermore, the allodynia was dependent upon background nociceptive
input as it disappeared upon complete cessation of the background pain. At this
point, vibration was once again described as innocuous (VAS = 0). Hence, a gentle
tactile stimulus capable of activating low-threshold mechanoreceptors, in the
presence of ongoing nociceptive input from muscle, triggered a crossover between
innocuous-touch and noxious-touch, i.e. aldynia. Fig. 1 shows the raw data from
one subject and the mean data (±SEM) of two subsequent 30 s periods of vibration (solid fills), interspaced with a 45 s interval, during muscle pain.

**Series 1. Allodynia abolished by conduction block of cutaneous C fibres**

Prior to intradermal anaesthesia, individual responses and the pooled mean data (±SEM) demonstrated that muscle pain significantly increased during vibration (Base: 100%; Vibration: 136.2 ± 8.6%, 135.5 ± 8.6%; \( P < 0.05; n = 11 \)) while all nerve fibres were intact. When local anaesthetic (Xylocaine 0.25%) was administered, and the C-fibre inputs from the vibration site consequently blocked, the VAS scores during vibration were not statistically different from the baseline muscle pain, i.e. the allodynia was abolished (Base: 100%; Vibration: 112.7 ± 11.3%, 110.3 ± 9.5%; \( P > 0.05; n = 11 \)).

The use of intradermal anaesthetic had no discernible effect on the intensity of baseline muscle pain (HS-induced), which confirms the intactness of muscle nociceptors (Arner et al. 1990; Mahns et al. 2006). The abolition of allodynia in the anaesthetized skin did not appear to be related to a decline in the capacity of the central nervous system to integrate these musculo-somatic inputs, as the allodynia was preserved in the adjacent non-anaesthetized skin where C fibres were intact (Base: 100%; Vibration: 134.4 ± 11.0%, 141.8 ± 12.6%; \( P < 0.05; n = 8 \)).

Therefore, a previously unidentified neural substrate in the C-fibre range with low-threshold mechanoreceptive properties seems to be involved in the generation of mechanical allodynia in glabrous skin (ANOVA: \( F = 5.595, P < 0.0001 \)).
Fig. 1 Vibration evoked reproducible increases in hypertonic saline-induced muscle pain. Continuous VAS recordings of a typical subject (a) and the mean VAS responses (b) to two successive vibration trains during muscle pain are shown (±SEM; n = 17; black: allodynia; unshaded: baseline muscle pain). Vibration (200 Hz-200 µm; 30 s), applied to the distal palmar pad of little finger (glabrous skin), evoked significant increases in muscle pain, i.e. allodynia. This effect persisted throughout the sustained phase of baseline pain as well as the decay phase that followed the termination of HS-infusion. Allodynia was short-lived, dissipating during the 45 s inter-vibration interval. In absence of muscle pain, all subjects described vibration as non-painful
Fig. 2a shows the mean vibration-evoked responses (±SEM) in duplicate sets for intact, anaesthetized and adjacent skin regions (3 treatment groups). The magnitude of allodynia was not significantly different ($P > 0.05$) in each treatment group.

**Series 2. Allodynia persists during conduction block of myelinated fibres**

During nerve compression, the amount of time taken to abolish, in entirety, vibrotactile and cool sensations was variable amongst subjects, but it invariably took effect within an hour (Nagi et al. 2011). While the myelinated afferents of ulnar nerve were blocked, infusion of HS evoked pain, which confirms earlier observations that C nociceptors alone can elicit HS-induced muscle pain (Laursen et al. 1999).

**Series 2a. Allodynia in glabrous skin mediated by low-threshold C afferents**

Individual responses and the pooled mean data (±SEM) demonstrate that the concurrent application of vibration to the glabrous skin of little finger (only C fibres intact) significantly increased muscle pain (Base: 100%; Vibration: 124.2 ± 4.4%, 121.3 ± 3.2%; $P < 0.001$; $n = 15$). During compression block, not only were the subjects blinded to auditory and visual cues but also they were unaware of any tactile cues. Despite that, they were able to reproducibly provide stimulus-locked increases in underlying muscle pain during vibration. The blockade of residual (C-fibre) input from the vibration site – within the innervation territory of blocked myelinated fibres – abolished the allodynic response to vibration (Base: 100%; Vibration: 98.4 ± 6.1%, 99.5 ± 6.0%; $P > 0.05$; $n = 15$). Conversely, in the adjacent non-anaesthetized skin where C fibres were intact, vibration evoked a significant increase in muscle pain (Base: 100%; Vibration: 126.5 ± 4.9%, 124.2 ± 4.3%; $P < 0.001$; $n = 11$). Hence, the blockade of myelinated fibres (C fibres intact) had no effect on allodynia, whereas
the blockade of unmyelinated cutaneous fibres abolished allodynia (ANOVA: $F = 11.06, P < 0.0001$). Fig. 2b illustrates the mean data (±SEM) in duplicate sets of successive vibration-evoked responses. The magnitude of allodynia was not significantly different ($P > 0.05$) in each treatment group.

**Series 2b. A CT fibre-like neural origin of allodynia in glabrous skin**

During muscle pain, gentle brushing of the glabrous skin of little finger (only C fibres intact) evoked a significant increase in the pain intensity (Base: 100%; Brushing at 1.0 cm s$^{-1}$: 135.9 ± 10.0%, 126.1 ± 5.6%; Brushing at 3.0 cm s$^{-1}$: 127.1 ± 5.5%, 133.9 ± 9.1%; $P < 0.05$; $n = 5$). This effect was reproducible, stimulus-locked and did not vary as a function of the brushing speed. Furthermore, consistent with the reproducibility of the brush-evoked effect, no changes ($P > 0.05$) were observed in the magnitude of allodynia during subsequent periods of brushing. Notably, the expression of allodynia in the glabrous skin in the absence of myelinated fibres was near identical to the CT-mediated allodynia in hairy skin (Nagi et al. 2011). The use of an intradermal anaesthetic to selectively block cutaneous C fibres was unfeasible, given the excitation of a much larger area of skin by a moving mechanical stimulus than punctate vibration. Nonetheless, brushing stimuli were applied at speeds with a demonstrated capacity to excite CTs, in addition to the generation of brush-evoked allodynia in the absence of myelinated fibres (ANOVA: $F = 9.233, P < 0.0001$). Mean data (±SEM) of two consecutive brush-evoked responses at each of the brushing speeds are shown in Fig. 2c.
Fig. 2 Alldynia in glabrous skin mediated by a functional homolog of C-tactile fibres. Mean touch-evoked responses (±SEM; black) were expressed as a percentage of the baseline muscle pain (unshaded). 

a Two successive vibration trains, applied to the distal palmar pad of little finger, reproducibly evoked significant increases in muscle pain (allodynia; \( n = 11 \)). Following C-fibre cutaneous block at the vibration site, alldynia was abolished. In the adjacent non-anaesthetized skin (proximal palmar pad) of little finger with all fibres intact, alldynia was preserved. 

b Significant vibration-evoked increases in muscle pain elicited during compression of myelinated fibres (ulnar nerve; \( n = 15 \)). Localized anaesthesia of cutaneous C fibres at the vibration site (glabrous skin of little finger) abolished alldynia. In the adjacent non-anaesthetized skin of little finger (C fibres intact) within the innervation territory of ulnar nerve, alldynia was preserved. Note that scaling is different in this figure. 

c Gentle brushing of digital glabrous skin (little finger; \( n = 5 \)) with absent myelinated fibres evoked a significant increase in muscle pain. The magnitude of alldynia was comparable between the two brushing speeds. * \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \)
Series 2c. Alloodynia comparable across spinal segments

During HS-infusion into FCU (ulnar nerve/mainly C8), the vibration-evoked effects within the same spinal segment (little finger, C8) were found to be comparable to the responses observed in a different spinal segment (index finger, C7). In both instances, the resulting allodynia was significant, reproducible and transitory, as it dissipated during the inter-vibration interval (Base: 100%; Vibration of little finger: 122.6 ± 4.9, 128.4 ± 5.9; Vibration of index finger: 125.5 ± 9.1, 127.5 ± 8.5; \( P < 0.05; n = 13 \)). Furthermore, no significant differences \( (P > 0.05) \) in the magnitude of allodynia were observed during repeated stimulations. In 5 of the 13 subjects tested, HS-infusion induced pain that extended beyond the wrist and in 3 subjects, referred to the little finger. However, there was no discernible change in the expression of vibration-evoked allodynia within (little finger) and beyond (index finger) the areas of referred pain. The expression of allodynia remained largely consistent across ulnar (only C fibres intact) and median (all fibres intact) innervation territories. These observations demonstrate the ubiquity of the allodynic effect of CLTMR-activation in the context of deep pain; allodynia elicited regardless of whether the stimulation site was located within the same or a different spinal segment and while the myelinated fibres were conducting or not (ANOVA: \( F = 7.296, P < 0.0001 \)). Mean data (±SEM) of successive vibration-evoked responses across spinal segments are shown in Fig. 3a.
Fig. 3 Allodynia comparable across spinal segments and skin types. Mean vibration-evoked responses (±SEM) were expressed as a percentage of the baseline muscle pain (unshaded). a Significant vibration-evoked increases in muscle pain (allodynia) elicited while the spinal projections from glabrous skin and FCU muscle overlapped or not (n = 13; black: allodynia in little finger; cross-hatched: allodynia in index finger). b Significant increases in muscle pain elicited, regardless of whether vibration was applied to the glabrous skin of little finger or the hairy skin of dorsal forearm (n = 9; black: allodynia in glabrous skin; cross-hatched: allodynia in hairy skin). In both a and b, the magnitude of CLTMR-mediated allodynia in little finger (only C fibres intact) was comparable to the allodynia evoked in index finger and dorsal forearm where all fibres were intact. * P < 0.05; ** P < 0.01
**Series 2d. Alloodynia comparable across skin types**

The mean data (±SEM) demonstrate that the expression of allodynia is identical in glabrous and hairy skin (Base: 100%; Vibration of glabrous skin: 123.6 ± 7.3, 127.8 ± 6.6; Vibration of hairy skin: 126.5 ± 9.0; 123.6 ± 8.8; \( P < 0.05; n = 9 \)). In both skin types, vibration evoked a significant and reproducible increase in muscle pain that abated before the onset of subsequent periods of vibration (ANOVA: \( F = 5.821, P < 0.0001 \)). This effect persisted regardless of the order of testing across skin types. No significant differences (\( P > 0.05 \)) were observed in the magnitude of allodynia within hairy and glabrous skin. These observations strongly suggest the existence of a functional homolog of CTs in the human glabrous skin that subserves mechanical allodynia. Mean data (±SEM) of successive vibration-evoked responses across skin types are shown in Fig. 3b.

**Discussion**

In this study, we have shown that ongoing muscle pain induced by infusion of hypertonic saline into the flexor carpi ulnaris muscle is enhanced by tactile stimulation of the hairy skin (forearm) as well as the glabrous skin (little and index fingers). By employing selective conduction blocks to the inputs arising from the glabrous skin of little finger, we observed (1) the abolition of allodynia during blockade (anaesthesia) of the unmyelinated cutaneous fibres; (2) the preservation of allodynia during blockade (compression) of the myelinated fibres. Furthermore, allodynia was not limited to an overlap in spinal projections from skin and muscle compartments, and was not a consequence of altered responsiveness within a referred pain region, as the vibration-evoked effects in a different spinal segment or a non-referred area were similar (cf. Klingon and Jeffreys 1958; Graven-Nielsen et al.
Moreover, there was no detectable difference in the expression of allodynia between glabrous and hairy skin. Thus, ongoing deep nociceptive input generates *surround* central sensitization; allodynia, which appears to be a manifestation of the central sensitized state, was comparable across spinal segments and skin types. Whether such a generalized central effect is unique to muscle pain, or also applies to ‘pain’ inputs from other body domains, warrants further exploration. Nonetheless, in pathological conditions, such as complex regional pain syndromes, pain does not necessarily spread in a somatotopic fashion such that regions beyond the innervation territory of the inciting lesion/nerve injury may be affected (Baron 2009). Based on our observations, the peripheral substrate of allodynia in glabrous skin constitutes a previously undefined class of C fibres with low-threshold mechanoreceptive properties that, at least functionally, seems to match the C-tactile fibres found in human hairy skin. In addition, this finding is consistent with the diminution of allodynia during mechanical stimulation of the glabrous skin in mice with absent VGLUT3-positive neurons (functionally defined CLTMRs: Seal et al. 2009)

Consistent with earlier predictions by Douglas and Ritchie (1957) that the processing of inputs arising from CLTMRs appears to occur at a subconscious level under normal conditions, we observed that vibration/brushing alone was always imperceptible during conduction block of myelinated fibres. This is also consistent with psychophysical and microneurography studies showing an unequivocal dependence of tactile perception on intact myelinated fibres (Hallin and Torebjörk 1976; Dellon 1980; Torebjörk and Hallin 1973). Furthermore, in spite of the characterization of a range of nociceptive C fibres, the absence of evidence on the existence of CLTMRs in humans lent further credence to the hypothesis that tactile
information was coded exclusively by myelinated afferents (Torebjörk 1974; Ochoa and Torebjörk 1989). However, we now know that low-threshold C mechanoreceptors provide a parallel tactile system in hairy skin (Vallbo et al. 1999). Although it is tempting to suggest that we are in the midst of a similar transition for CLTMRs in glabrous skin, this remains a matter for conjecture. Nonetheless, the finding of this paper – the first of its kind – shows that the activation of mechanoafferents, within the C-fibre range, in the glabrous skin by vibration/brushing during sustained nociceptive input from the muscle generates a perceptual effect in the form of allodynia. In addition to the prerequisite of ongoing nociceptive input, we observed that the onset of allodynia tended to be delayed by ~10-15 s suggesting a dependence on slow-conducting fibres and the possible need for temporal summation. Once initiated, the increased pain rating ‘overshot’ the stimulus duration (Torebjörk et al. 1992). However, allodynia was short-lived, as it invariably dissipated during the 45 s interval between successive periods of tactile stimulation. These observations are consistent with the response properties of low-threshold mechanosensitive nerve endings (Zotterman 1939; Douglas and Ritchie 1957). Furthermore, it has recently been proposed that Neurokinin-1 (NK-1) receptors are located on CLTMRs (amongst a range of receptor systems) and, more importantly, mechanical allodynia evoked in glabrous skin can be abolished by a peripheral NK-1 antagonist (Zhang et al. 2008; Carlton et al. 1996). Despite these similarities, it remains to be ascertained whether the responsible C-fibre class terminates as free endings in the glabrous skin, or is consistent with other types of C-fibre innervations, e.g. those associated with Meissner corpuscles. Nevertheless, these observations contribute to our current understanding of cross-modality,
musculo-somatic interactions by revealing a distinct role for low-threshold C-fibre inputs in the production of allodynia.

It is now widely accepted that allodynia is mediated by changes in the central nervous system (e.g. Simone et al. 1991; Torebjörk et al. 1992). Numerous reports quite logically linked the production of mechanical allodynia to the activation of low-threshold large myelinated fibres (Campbell et al. 1988; Gracely et al. 1992; Koltzenburg et al. 1994). The involvement of these fibres, which had been consistently shown to evoke non-painful tactile sensations following electrical intraneural microstimulation (INMS), appeared to be case-specific as concurrent activation during ongoing nociceptive input (central sensitized state) was subsequently reported as painful (Torebjörk et al. 1992). Although the INMS experiments have established the role of large myelinated afferents in allodynia, the question of whether low-threshold unmyelinated afferents contribute as well has remained unaddressed. Furthermore, the expression of allodynia in general, and its somatotopic representation in particular, was mainly examined in the context of cutaneous pain. Moreover, it is often pointed out that the induction, or sustenance, of large fibre-mediated allodynia necessitates an elaborate reorganization at the dorsal horn level (Woolf et al. 1992; Woolf and Salter 2000). However, recent evidence suggests that such reorganization is in fact minimal (Bao et al. 2002). Notably, in our experimental paradigm, we can reversibly produce alldynia over a brief period of HS-infusion, which conforms to the findings in animal models showing activation of superficial dorsal horn neurons by CLTMR-mediated inputs from hairy and glabrous skin (Seal et al. 2009; Andrew 2010). Furthermore, low-threshold C fibres provide sizeable terminations to the substantia gelatinosa, which has been proposed as a
modulator of inputs from skin, muscle and viscera (Light and Perl 2003). Moreover, it is often argued that low-threshold C-fibre inputs project from spinal lamina I neurons to the posterior portion of the ventromedial thalamic nucleus (VMpo: Craig 2003; Andrew 2010). Inputs from the VMpo project to the dorsal posterior insular cortex (dpINS), also termed the interoceptive cortex (Craig 2003; Olausson et al. 2002).

The lamina I-VMpo-dpINS system, which appears to be comparable for inputs from hairy and glabrous skin, can provide an underlying mechanism for allodynia in our paradigm: mechanoreceptive impulses transmitted by low-threshold C fibres converge, at a central level, with nociceptive input from HS-activated muscle receptors, thereby eliciting a perceptual response that lacks compliance with stimulus characteristics (allodynia). However, whether the mechanoreceptive inputs from low-threshold C fibres from hairy and glabrous skin undergo a similar mechanism of central integration/processing warrants further exploration. Nonetheless, in the context of allodynia, the perceptual outcome of the activation of low-threshold C fibres innervating either type of skin appears remarkably similar. These findings should alert investigators to search for CLTMRs in human glabrous skin, given the potential clinical implications for understanding the sensory-perceptual impairments that manifest in often therapeutically intractable pain conditions and neurological disorders.
Acknowledgements

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Disclosures

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References


Mackenzie RA, Burke D, Skuse NF, Lethlean AK (1975) Fibre function and perception during cutaneous nerve block. J Neurol Neurosurg Psychiatry 38 (9):865-873


PAPER III

Modified version presented in J Pain format
C-TACTILE FIBERS CONTRIBUTE TO CUTANEOUS ALLODYnia AFTER ECCENTRIC EXERCISE

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Running title: C-tactile fibers mediate allodynia in DOMS

Keywords: C fiber; allodynia; hairy skin; eccentric exercise; delayed onset muscle soreness; pressure pain threshold; intradermal anesthesia

All experiments were performed at the University of Western Sydney, School of Medicine, Sydney NSW, Australia.
Abstract
We recently showed that during acute muscle pain, C-tactile (CT) fibers mediate allodynia in healthy human subjects. In this study, we pursued the following questions: do CTs contribute to allodynia observed in delayed onset muscle soreness (DOMS)?; and is CT-mediated allodynia reproducible in a clinical pain-state? In 30 healthy subjects, DOMS was induced in anterior-compartment muscles of leg by repeated eccentric contractions. DOMS was confirmed by mapping the emergence of tender points (decreased pressure-pain thresholds, PPTs). Furthermore, we measured PPTs in a clinical subject who presented with activity-triggered heel-pain, albeit there was no resting pain. Cutaneous vibration (sinusoidal; 200 Hz-200 μm) – an otherwise innocuous stimulus – was applied to anterolateral leg before exercise, during DOMS and following recovery from DOMS. The peripheral origin of allodynia was determined by employing conduction blocks of unmyelinated (intradermal anesthesia) and myelinated (nerve compression) fibers. In DOMS-state, there was no resting pain, but vibration reproducibly evoked pain (allodynia). The blockade of cutaneous C fibers abolished this effect, whereas it persisted during blockade of myelinated fibers. In the clinical subject, without exposure to eccentric exercise, vibration (and brushing) produced a cognate expression of CT-mediated allodynia. These observations attest to a broader role of CTs in pain processing.

Perspective
This is the first study to demonstrate the contribution of C-tactile (CT) fibers to mechanical allodynia in exercise-induced as well as pathological pain-states. These findings are of clinical significance, given the crippling effect of sensory impairments on the performance of competing athletes and patients with chronic pain and neurological disorders.

Abbreviations CT, C-tactile; DOMS, delayed onset muscle soreness; PPT, pressure-pain threshold; VAS, visual analog scale.
Introduction

Within the somatosensory system, stimulus encoding occurs peripherally at the receptor and is transmitted to higher-order neurons in a manner that allows us to readily differentiate between robust-to-subtle variations in a wide range of stimuli. However, in clinical conditions, we often observe a perceptual response that lacks compliance with stimulus characteristics, e.g. touch-evoked pain. This phenomenon is known as *allodynia*. We recently showed that a rather abstruse class of low-threshold unmyelinated mechanoreceptors, termed C-tactile (CT) fibers, mediates allodynia during acute muscle pain. Conversely, in absence of background pain, the activation of CT fibers correlates with a diffuse sensation of pleasant touch. Therefore, background nociceptive input appears to play a crucial role in the production of CT-mediated allodynia. Nonetheless, it remains to be elucidated whether a ‘perceptual’ level of pain is necessary to unmask the central effects of CT-fiber inputs, or can the allodynic response be elicited by sub-perceptual events. Furthermore, whether the expression of CT-mediated allodynia can be reproduced in a more persistent, or clinical, pain-state remains to be tested.

To pursue these questions, we employed a natural form of muscle damage, termed delayed onset muscle soreness (DOMS), which can be evoked by unaccustomed eccentric exercise (weight-bearing during muscle lengthening); downhill walking, for example. Following exercise, muscle soreness gradually develops; it usually peaks at 24–48 h post-exercise and invariably subsides within a week. Although there need not be any resting pain following the onset of DOMS, tenderness is often reported to palpation, contraction and stretch in the affected muscles. Muscle tenderness can be quantified by mapping the emergence of tender points (decreased
pressure-pain thresholds, PPTs) within the exercised muscles.\textsuperscript{64,65} The mechanism of DOMS is poorly understood. Based on animal work, it was postulated that muscle fiber degeneration and necrosis were the primary features of eccentric exercise-induced damage.\textsuperscript{15,16,31} However, recent studies in humans have indicated that myofiber degeneration and necrosis are not characteristic of DOMS,\textsuperscript{67} and that it is in fact the ‘adaptive remodelling’ of myofibers that has been considered to represent damage.\textsuperscript{68} In addition, DOMS has been associated with inflammation with and without muscle damage as a primary event.\textsuperscript{35,38,52}

Both muscle mechanoreceptors\textsuperscript{2,64,65} and nociceptors\textsuperscript{19,38,60} have been implicated in delayed soreness after exercise. Based on converging evidence, it is posited that the symptoms of DOMS, e.g. a fall in mechanical threshold of exercised muscles, are an expression of central sensitization – in particular, changes at the level of the superficial dorsal horn.\textsuperscript{61} What’s intriguing is the dearth of information on the central effects of cutaneous inputs during DOMS, particularly given that CT-fiber endings terminate densely in the superficial laminae of dorsal horn.\textsuperscript{1,55,58} Furthermore, CT-mediated inputs have been shown to project to the limbic-emotional regions of the brain.\textsuperscript{5,11} Whether the activation of CT fibers expresses allodynia during delayed onset muscle soreness formed the focus of this study. DOMS was induced by eccentric exercise of the anterior-compartment muscles of the leg. In addition, we investigated the contribution of CTs to allodynia in a clinical subject who reported no resting pain, but physical exertion evoked recurrent bouts of bilateral heel-pain and induced a reversible alteration in gait (toe-walking). Given that the postural adjustment, exhibited by the subject, mimics eccentric contractions of the anterior muscles of leg, we need not induce muscle damage experimentally in order to
produce DOMS. Akin to our earlier work,\textsuperscript{43} nerve conduction blocks were employed in order to determine the peripheral substrate of allodynia.

Some of the results have been published in abstract form.\textsuperscript{41}

\textbf{Materials and Methods}

Thirty healthy human subjects (21 males & 9 females, aged 18-40 years) and a chronic pain patient (male, 19 years) took part in this study. Informed consent was obtained from each subject in writing. All experiments were approved by the UWS Human Research Ethics Committee (approval number: H9190) and conformed to the principles of the Declaration of Helsinki. In each subject, the perceptual response to cutaneous vibration was examined just prior to eccentric exercise and once DOMS had set in. Furthermore, the peripheral neural substrate for realizing any change in the quality of vibration across pre-exercise and DOMS states was determined by employing nerve conduction blocks. While performing these tests, subjects sat comfortably on a chair with both legs horizontally stretched out and supported on both sides.

\textit{Cutaneous vibration}

A circular Perspex (Plexiglas) probe with a rounded 4 mm diameter tip was placed perpendicular to the skin surface, overlying the anterior-compartment muscles of leg, without compressing the underlying structures. Subjects were questioned to ensure that there was no discomfort at the site of contact. The probe was attached to a feedback-controlled sinusoidal stimulator.\textsuperscript{37} The frequency (200 Hz) and amplitude (200 $\mu$m) parameters were chosen, as the resulting stimulus is normally innocuous
and is capable of activating a range of cutaneous and sub-cutaneous mechanoreceptors. Each period of vibration lasted 30 s and was repeated at 45 s intervals in order to avoid desensitization of the activated fiber classes. The inter-stimulus interval of 45 s conforms to the recovery time for CT-fiber fatigue in humans (~30 s), in addition to the stimulation intervals followed in recent psychophysical and microneurography studies on this afferent class. Furthermore, such an interval provided sufficient time to perform sensory tests in order to track the progression of nerve conduction blocks. Moreover, it is consistent with our earlier findings in an acute model of pain, which showed that the CT-mediated response can ‘overshoot’ the stimulus duration, but invariably dissipates within a 45 s interval (see also). Brown noise was delivered through headphones to ensure that auditory cues associated with the vibrotactile stimulator were not provided.

**Delayed onset muscle soreness**

DOMS was induced in the anterior-compartment muscles of the lower limb by slow, repeated plantarflexions of the foot (10 times per set; 9 sets in total). The generation of DOMS was confirmed by systematically mapping the emergence of tender points (decreased pressure-pain thresholds, PPTs) within the exercised muscles using a force gage with a 1 cm² rubber tip (Pain Test algometer, Wagner Instruments, Greenwich, CT, USA). To ensure muscle loading during plantarflexion, the entire body weight was borne on the experimental leg (single-leg stance) with the subject standing on a 15 cm high metal platform at hind-foot level and inclining towards the wall. A 2.25 kg weight belt was strapped to the distal leg in order to apply a greater load on the stretched muscles. Prior to the induction of DOMS, a 2×2 cm grid comprising of 30 points was drawn on the skin overlying the targeted muscles,
as shown in Fig. 1. The minimal pressure required to produce ‘detectable’ pain was measured at each grid point in a randomized pattern across a range of intensities (5-30 N, in multiples of 5). The stimulus duration was ~1 s and an interval of ~5 s was provided before applying stimulus to another spot. Each point was tested prior to and 24 h after a bout of eccentric exercise (Fig. 1). In cases where an appreciable drop in PPTs was not observed, i.e. more than half of the grid points expressing a pain threshold beyond our testable range (i.e. greater than 30 N), a second bout of eccentric exercise was performed 24 h after the initial bout and the PPTs determined on the following day. Vibration was applied to the skin overlying the control (> 30 N) and tender (< 30 N) spots of the exercised leg in order to document the emergence of allodynia.

Assessment of allodynia

The intensity of vibration-evoked pain (allodynia) was measured on a visual analog scale (VAS) ranging from 0 (no pain) to 10 (worst pain). Subjects were instructed to rotate a potentiometer, the signals from which were recorded on a computer (Spike2 version 6.05a, Cambridge Electronic Design, Cambridge, England). The perceptual crossover in the quality of vibration prior to exercise and during DOMS was quantified using the Touch Perception Task (TPT), which consists of a mix of sensory and emotional attributes (40 in total). Furthermore, the quality of vibration-evoked pain in the DOMS-state was captured on the McGill Pain Questionnaire (MPQ). By administering the TPT and the MPQ, we explored whether the activation of CT afferents is associated with a unique quality of sensation.
Figure 1. A map of pressure-pain thresholds in the anterior-compartment muscles of the leg prior to (left) and 24 h after (right) a bout of eccentric exercise. A 2×2 cm grid consisting of 30 points was drawn on the skin overlying the targeted muscles. The minimal pressure required to produce detectable pain was measured at each grid point by applying a calibrated force gauge across a range of intensities (5-30 N). Each grid point is represented by a circle: the thicker the circumference, the lower the threshold. In this subject, more than 50% of the grid points were reported as painful at pressure intensities as low as 15 N. Hence, a second bout of eccentric exercise was not performed.
Nerve conduction blocks

As described in our earlier publication,\textsuperscript{43} the peripheral substrate of vibration-evoked allodynia was determined by employing nerve conduction blocks. In experimental Series I, after recording responses to at least three consecutive vibration trains, 0.2-0.4 ml of a local anesthetic (Xylocaine: 0.25\%) was injected into the skin region stimulated by vibration. This was aimed at preferentially blocking the C-fiber inputs from a \~2 cm region of skin overlying the exercised muscles whilst preserving deep/remote C-fiber inputs and all the large-fiber inputs. The effectiveness of the C-fiber blockade was ascertained by the loss of perceptiveness to warm stimuli (\~40\(^\circ\)C, brass rod in contact with the skin for 5 s), whereas the preservation of vibration (20 & 200 Hz; 200 \(\mu\)m) and cool (\~15\(^\circ\)C, brass rod in contact with the skin for 5 s) sensations was taken for the intactness of myelinated fibers.\textsuperscript{24,36,62} Vibration was applied within the anesthetized skin (C fibers blocked) and in the adjacent non-anesthetized skin with a full complement of sensory fibers. Indeed, we have shown that an intradermal injection of local anesthetic has no perceptual bearing on the activity of muscle nociceptors or those receptors located in the vicinity of joints and bones.\textsuperscript{37,43}

In experimental Series II, which was performed subsequent to Series I, a compression block of the sciatic nerve was employed by placing a metal bar just distal to the ischial tuberosity in order to preferentially block the impulses propagated by myelinated fibers.\textsuperscript{24,57,64} To determine the effectiveness of the compression block, somatosensory sensibility within the innervation territory of sciatic nerve was compared with skin regions on the medial aspect of the exercised leg, which is innervated by the femoral nerve, and the contralateral leg.\textsuperscript{13} The
abolition of vibration and cool sensations within the sciatic innervation territory was taken as confirmation of an effective blockade of myelinated fibers, while the preservation of warm sensibility affirmed the integrity of C fibers. Once the block had taken effect, successive vibration-evoked responses were recorded within the innervation territory of sciatic nerve (only C fibers intact). Akin to Series I, a low-dose intradermal anesthetic was used in order to block the residual cutaneous input (C fibers only) from the vibration site. Thenceforth, VAS responses to vibration were recorded within the anesthetized region with all the cutaneous input blocked as well as the adjacent non-anesthetized skin with C fibers intact. While performing the sensory tests to determine the progression/effectiveness of conduction blocks, we shielded the subjects from visual and auditory cues.

**Chronic pain patient**

The clinical subject, Z.S., suffers from activity-triggered bilateral heel-pain that appeared impromptu about 2 years ago. Physical exertion induces recurrent bouts of bilateral heel-pain that invariably outlast the duration of the activity, albeit there is no resting pain. Based on an ultrasound examination of both feet, Z.S. was diagnosed as having ‘plantar fasciitis’. Furthermore, during a bout of pain, Z.S. modifies his gait by walking on the toes, presumably as a pain-avoidance response. The altered gait lengthens the anterior-compartment muscles of the lower limb, akin to the loaded eccentric contractions that induce DOMS. Any experimentally induced muscle damage was deemed inessential given that conditions similar to DOMS already existed, as indicated by the detection of multiple tender points (> 50%) in the anterior-compartment muscles of the leg.
Consistent with the healthy controls, vibration was applied to the skin overlying the anterolateral leg. Furthermore, high-precision brush strokes were applied to this region of skin at speeds of 1.0 and 3.0 cm s\(^{-1}\) as slow-moving, low-force, mechanical stimuli have been extensively used for the activation of CTs.\(^{34,47,48}\) Akin to our earlier observations in a non-clinical sample,\(^{43}\) a paintbrush (0.7 cm thick and \(\approx 7.5\) cm wide) of goat’s hair (3.0 cm long) was swept bi-directionally – perpendicular to the skin surface along a proximo-distal and disto-proximal direction – through a stroke of 10.0 cm plus a 1.0 cm turnaround at each end. The stimuli were delivered using a linear motor on a 3D Gantry system (Baldor Australia Pty Ltd), which was under feedback of a PMAC motion controller (Delta Tau Data Systems, Inc.). In conformity with vibratory stimulation, the brush strokes were applied for 30 s with an inter-stimulus interval of 45 s.

Based on a clinical examination of the site of tactile stimulation, the following aspects of the somatosensory system were found to be intact: thermal sensibility, static/dynamic touch sensibility, tactile localization and directional sensibility. In addition to markedly low PPTs in the anterior muscles of leg, Z.S. also displayed a heightened response to 5% hypertonic saline – infused into the tibialis anterior muscle – relative to healthy controls.\(^{43}\) As detailed in \textit{Series I} and \textit{II}, differential nerve blocks were used in order to determine the peripheral fiber contributions to clinical allodynia. At the time of these experiments, the subject was not taking any medication.
**Statistical analysis**

Data analyses were performed using GraphPad Prism (version 5.04 for Windows, GraphPad Software, San Diego, CA, USA) and are presented as mean and standard error of the mean (±SEM). In each healthy individual, the VAS response to vibration during DOMS was compared to the pre-exercise response (base) or, in case of the clinical subject, the baseline just prior to tactile stimulation. In each subject, triplicate base and vibration values were analyzed as independent, sequential events. Significant changes were detected using a one-way repeated measures analysis of variance (ANOVA). Where significant differences were indicated ($P < 0.05$), individual groups were compared using a Newman-Keuls multiple comparison test. The raw PPT data were analyzed with two-way ANOVA with factors time (pre-exercise & post-exercise) and group (responders & non-responders) in order to determine the relationship between PPT changes (number of tender spots) and the production of DOMS. Bonferroni post-tests were used to compare individual groups.

**Results**

Prior to eccentric exercise, all the subjects ($n = 30$) reported vibration as non-painful (VAS = 0) and devoid of any distinct emotional attribute – ‘vibrating’ was the only descriptor ascribed to the resulting sensation from the TPT. Following eccentric exercise, DOMS failed to elicit in 12 subjects (non-responders), even when a second bout of exercise was performed. That is, less than half of the grid points (37%) were reported as painful within our testable range (5-30 N). In the remaining 18 subjects (responders), a clear-cut drop in PPTs was observed with more than half of the grid points (~71%) reported as painful following eccentric exercise, i.e. DOMS
established (Table 1 & Fig. 2). Furthermore, 75% of the *responders* reported tenderness to stretch and contraction in the exercised muscles, as against only 40% of the *non-responders*. Notably, the mean data (±SEM) demonstrate that the post-exercise PPT values in the *non-responders’* group were not statistically different (*P* > 0.05) from the pre-exercise PPT values in the *responders’* group (Fig. 2). It appears that the background level of muscle soreness, i.e. number of tender spots prior to exercise, determines, in part at least, whether DOMS will develop or not. When we compared the pre-exercise PPT values of subjects where DOMS elicited relative to those where it failed to elicit, the pain threshold is consistently seen to be 5-10 N higher in the latter (Table 1). The results of the two-way ANOVA indicate that the interaction term (*F* = 11.12; *P* < 0.0001), the effect of time (pre- & post-exercise: *F* = 93.21; *P* < 0.0001) and the effect of group (*responders* & *non-responders*: *F* = 16.08; *P* < 0.0001) are all significant. The Bonferroni post-tests reveal that the number of post-exercise tender spots in the *responders’* group is significantly greater (*P* < 0.001) – across a range of pressure intensities as low as 15 N – from the pre-exercise values in that group in addition to the pre- and post-exercise values in the *non-responders’* group (Table 1 & Fig. 2). In the *responders’* group, a second bout of exercise was performed in 12 of the 18 subjects; although the drop in PPT values tended to be slightly more pronounced relative to the initial bout, it did not differ significantly (*P* > 0.05). Therefore, the number of exercise bouts was not factored into the results (see also\textsuperscript{8,9}).
Table 1. Mean Pressure-pain Threshold (Number of Sore Spots) Prior to and Following Eccentric Exercise (±SEM; n = 30)

<table>
<thead>
<tr>
<th>Group</th>
<th>Session</th>
<th>5 N</th>
<th>10 N</th>
<th>15 N</th>
<th>20 N</th>
<th>25 N</th>
<th>30 N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responders (n = 18)</td>
<td>Pre-exercise</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.3</td>
<td>2.3 ± 0.8</td>
<td>4.2 ± 1.3</td>
<td>6.7 ± 1.8</td>
<td>9.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Post-exercise</td>
<td>1.4 ± 0.4</td>
<td>4.4 ± 0.9</td>
<td>9.6 ± 1.5</td>
<td>13.7 ± 2.0</td>
<td>18.2 ± 1.9</td>
<td>21.4 ± 1.5</td>
</tr>
<tr>
<td>Non-responders (n = 12)</td>
<td>Pre-exercise</td>
<td>0.0 ± 0.0</td>
<td>0.3 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>1.8 ± 0.9</td>
<td>2.5 ± 1.2</td>
<td>4.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Post-exercise</td>
<td>0.1 ± 0.1</td>
<td>0.8 ± 0.6</td>
<td>2.0 ± 0.9</td>
<td>4.7 ± 1.6</td>
<td>7.3 ± 1.9</td>
<td>11.1 ± 2.2</td>
</tr>
</tbody>
</table>
Figure 2. Differential effects of eccentric exercise on pressure-pain thresholds. In the 18 subjects where DOMS elicited (responders), the mean data (±SEM) demonstrate that the emergence of post-exercise sore spots was significantly different – across a range of pressure intensities as low as 15 N – relative to the pre-exercise values in addition to the pre- and post-exercise values in the non-responders’ group (n = 12). In contrast, the post-exercise PPT values in the non-responders’ group were statistically indistinguishable from the pre-exercise PPT values in the responders’ group. *** $P < 0.001$
**Allodynia elicited by vibration in DOMS-state**

During the DOMS-state (i.e. responders), a perceptible crossover between sensations was reported where an otherwise emotionally inert vibrotactile stimulus was associated with overtly negative qualities of affection on the TPT. More than 85% of the subjects described the sensation during vibration as ‘discomfort’, ‘irritating’, ‘prickly’; ~71% reported it as ‘sharp’; and ~57% linked it to ‘burning’. This effect (allodynia) was significantly different from the pre-DOMS vibration-evoked response, or the baseline during DOMS, both of which were naught on the pain scale (base: 0.0 ± 0.0; vibration: 1.6 ± 0.2, 1.6 ± 0.2, 1.7 ± 0.2; P < 0.001; n = 18; Fig. 3). Furthermore, allodynia was reproducible and stimulus-locked, i.e. it invariably ebbed to the baseline (VAS = 0) during the inter-stimulus interval. Where possible, vibration was applied to a control spot (> 30 N) as well, which evoked a cognate expression of allodynia.

**Series I. Allodynia abolished by conduction block of unmyelinated fibers**

Following the use of a low-dose intradermal anesthetic to selectively block C-fiber inputs, vibration-evoked allodynia was abolished in the anesthetized skin (base: 0.0 ± 0.0; vibration: 0.4 ± 0.1; 0.4 ± 0.1; 0.4 ± 0.1; P > 0.05; n = 18; Fig. 3). In the adjacent non-anesthetized skin with all fibers intact, allodynia was preserved (base: 0.0 ± 0.0; vibration: 1.5 ± 0.2; 1.6 ± 0.3; 1.5 ± 0.2; P < 0.001; n = 18; Fig. 3). Furthermore, each vibration-evoked response prior to the blockade of C fibers or in the adjacent non-anesthetized skin (all fibers intact) was significantly different (P < 0.001) from the vibration-evoked response within the anesthetized skin. Moreover, the magnitude of allodynia within each specific region (intact, anesthetized and adjacent) was not significantly different (P > 0.05).
Figure 3. Allodynia abolished by conduction block of unmyelinated fibers. Mean vibration-evoked responses during DOMS (VAS > 0; ±SEM; n = 18; gray) were compared to the pre-exercise responses (VAS = 0). In absence of resting pain, allodynia elicited during successive periods of vibration. This effect was significant, reproducible and stimulus-bound. Allodynia was abolished by the blockade of C fibers in the skin region stimulated by vibration. In the adjacent non-anesthetized skin with all fibers intact, allodynia was preserved. *** P < 0.001
These observations indicate that the role of CT fibers in pain processing is not just limited to the acute models of pain, but also extends to the more persistent pain-states such as DOMS (ANOVA: $F = 29.07, P < 0.0001$). In addition, the results of the MPQ complement these findings with a broad range of pain-descriptors that were ascribed to CT-mediated allodynia. Each of the following descriptors was reported by at least 15% of the subjects in compliance with the mean incidence level: ‘pricking’; ‘drilling’; ‘sharp’; ‘pressing’; ‘dull’; ‘sore’; ‘tender’; ‘annoying’; ‘spreading’; ‘radiating’; and ‘tight’. To our knowledge, this is the first study to reveal the perceptual effects of CT-fiber activation, given that earlier work on CTs was either performed on non-human animals or the experimental paradigms were limited by the persistence of overt background pain.

**Series II. Allodynia persists during conduction block of myelinated fibers**

In 8 of the 18 subjects where DOMS elicited, a compression block was employed in order to determine the contribution of myelinated fibers to allodynia. Prior to the blockade of myelinated fibers, vibration reproducibly evoked pain that was significantly different from the VAS baseline (base: 0.0 ± 0.0; vibration: 1.7 ± 0.3, 1.6 ± 0.3, 1.6 ± 0.3; $P < 0.001$; $n = 8$; Fig. 4). This effect persisted following the blockade of myelinated fibers (base: 0.0 ± 0.0; vibration: 1.1 ± 0.4, 1.1 ± 0.4, 1.1 ± 0.4; $P < 0.05$; $n = 8$; Fig. 4). The expression of allodynia was reproducible and short-lived, as it always dissipated during the inter-stimulus interval. Furthermore, the magnitude of VAS responses (allodynia) prior to and following compression block was statistically indistinguishable. Interestingly, the magnitude of alldynia was also comparable to our earlier work using a hypertonic saline-based acute model of muscle pain where a background pain rating was necessary to generate a CT-mediated allodynic response to concurrent vibration and brushing stimulation.
**Figure 4.** Allodynia persists during conduction block of myelinated fibers. In 8 of the 18 subjects where DOMS elicited, a compression block of myelinated fibers was employed. Mean vibration-evoked responses show significant VAS changes (±SEM; n = 8; gray) while the myelinated fibers were conducting or not. The magnitude of allodynia prior to and following compression block was statistically indistinguishable. *P < 0.05; **P < 0.01; ***P < 0.001
These observations provide further evidence that C-tactile fibers contribute to allodynia following eccentric exercise (ANOVA: $F = 8.634$, $P < 0.0001$). In 4 of the 8 subjects where a compression block was employed, we also used an intradermal anesthetic in order to abolish the residual cutaneous input from the vibration site within the territory of blocked myelinated fibers. Consistent with Series I results, the blockade of C fibers in the skin abolished allodynia (base: $0.0 \pm 0.0$; vibration: $0.0 \pm 0.0$). Vibration-evoked allodynia appears to be a complement of inputs from activated CTs in the skin and those nerve fibers presumably located within the muscle that mediate exercise-induced muscle soreness, as vibration applied upon cessation of DOMS (one week after exercise) was reported as non-painful.

**C-tactile fibers contribute to clinical allodynia**

In the clinical subject, vibration and brushing reproducibly evoked a comparable, or amplified, expression of CT-mediated allodynia, without the need to perform eccentric exercise, as shown in Fig. 5. Given that the magnitude of the effect was largely consistent across triplicate stimulations, and for the sake of clarity, the corresponding VAS scores were collapsed into an averaged pain rating (base: $0.0 \pm 0.0$; vibration: $6.5 \pm 0.6$; brushing at $1.0 \text{ cm s}^{-1}$: $2.0 \pm 0.1$; brushing at $3.0 \text{ cm s}^{-1}$: $3.1 \pm 0.2$). Consistent with healthy subjects, the blockade of C fibers markedly reduced the intensity of vibration-evoked allodynia (base: $0.0 \pm 0.0$; vibration: $1.1 \pm 0.6$), whereas in the adjacent non-anesthetized skin, allodynia remained intact (base: $0.0 \pm 0.0$; vibration: $5.1 \pm 1.1$). Given that a much larger area of skin was excited by brush strokes as against punctate vibration, the use of an intradermal anesthetic was deemed infeasible. Notably, vibration- and brush-evoked allodynia persisted
following the blockade of myelinated fibers (base: 0.0 ± 0.0; vibration: 3.9 ± 0.3; brushing at 1.0 cm s⁻¹: 2.3 ± 0.3; brushing at 3.0 cm s⁻¹: 3.2 ± 0.3).

Akin to healthy subjects, the TPT results for vibrotactile stimulation revealed a distinct crossover between innocuous-touch (pre-exercise) and noxious-touch (during DOMS): in the former, ‘vibrating’ was the only attribute chosen, whereas in the latter, in addition to ‘vibrating’, ‘discomfort’ and ‘irritating’ were also used. Furthermore, the following descriptors were ascribed to clinical allodynia on the MPQ: ‘throbbing’; ‘drilling’; ‘pinching’; ‘sore’; ‘tiring’; ‘annoying’; ‘spreading’. With the exception of ‘vibrating’, all the other attributes were reproducibly linked to allodynia, regardless of whether myelinated fibers were blocked or not. Hence, CT-mediated allodynia appears to be a generalized phenomenon that can be expressed in pathological conditions, including sites remote from the origin of pain.

**Discussion**

In this study, we showed that a normally innocuous vibrotactile stimulus, applied to the skin, is perceived as painful (allodynia) during delayed onset muscle soreness. This observation indicates that cutaneous allodynia is an expression of the central sensitized state, which, in this instance, appeared to be an effect of repeated eccentric contractions. Furthermore, we demonstrated the emergence of allodynia in absence of resting pain, hence the presumed plastic changes that elicit allodynia can be realized by sub-perceptual events. In our study, allodynia was abolished during the conduction block of unmyelinated cutaneous fibers, whereas it remained intact during the blockade of myelinated fibers. This is the first demonstration of the role of CT fibers in pain processing in conditions other than acute muscle pain.⁴³
Figure 5. C-tactile fibers contribute to clinical allodynia. In the right panel, a map of pressure-pain thresholds in the anterior-compartment muscles of the leg is shown. The emergence of a remarkable number of sore spots (> 50%) without exposure to eccentric exercise represent a DOMS-like state of altered processing in the clinical subject. In the left and middle panels, averaged vibration- and brush-evoked responses (±SEM) are shown prior to and following differential nerve blocks. While all fibers were intact, vibration and brushing (at both speeds) reproducibly evoked allodynia of a heightened intensity relative to healthy subjects. The vibration-evoked effect was markedly reduced by employing a C-fiber cutaneous block, whereas in the adjacent non-anesthetized skin (all fibers intact), allodynia was preserved. Furthermore, vibration- and brush-evoked allodynia persisted regardless of whether myelinated fibers were conducting or not.
In the clinical subject, vibration evoked a comparable, or amplified, expression of allodynia; an effect that was also observed in response to slow, gentle brushing. Although pain-free at the time of the experiment, Z.S. experienced frequent bouts of bilateral heel-pain (plantar fasciitis) triggered by physical exertion. The pain invariably outlasted the duration of the activity and induced a reversible alteration in gait (toe-walking). Consistent with the latter observation, and the detection of multiple tender points (as low as 10 N), the muscles of the anterior leg appeared to be in a chronic state of trauma. Furthermore, the production of pain during vibration and brushing, without being subjected to the exercise protocol, suggests that the central mechanisms were primed to elicit an allodynic response to CT-fiber inputs. These observations demonstrate that assessment of both cutaneous and muscle sensibility may be useful in disentangling the source of pain from the events that intensify it.

It has recently been shown that repeated activation of muscle nociceptors can re-map the localization of muscle pain to adjacent structures.\textsuperscript{53} In this study, we see a similar mismatch between the origin of pain and its perceptual localization. Allodynia was poorly localized with pain being reported within the exercised muscle, at the skin region stimulated by vibration or somewhere in the distal leg (including ankle) without a distinct location. Irrespective of the localization pattern, allodynia was abolished following blockade of C fibers at the vibration site. We know very little about the capacity of CT-mediated inputs to localize stimulus/sensation with the exception of two large-fiber neuropathy patients who were able to detect the presence, albeit faintly, of light tactile stimuli, but failed to detect the direction of movement.\textsuperscript{10,47,48} Similarly, based on the failure of a single large-fiber deafferented patient to detect a rapidly moving stimulus, it was inferred that the mechanical
sensitivity of CT fibers is limited to the slowly moving forms of stimuli. However, the warmth thresholds of these patients were reported outside the normal range of detection, which indicates at least a partial impairment of the C-fiber system.

Based on animal work, we know that the responsiveness of CT fibers to moving stimuli depends on the rate of motion across the receptive field and the interval between successive stimuli. Notably, a progressive fall, or ‘fatigue’, has been observed at an inter-stimulus interval of less than 30 s. In healthy human subjects, Vallbo and colleagues demonstrated that the initial spike activity in CTs remained fairly consistent during repetitive stimulation; a finding suggesting a reduced susceptibility of CTs to fatigue in humans. Furthermore, in response to sustained indentation, a subset of CTs displayed a recovery of discharge frequency after an initial phase of adaptation – a phenomenon termed ‘delayed acceleration’. However, the typicality and significance of CT-fiber activity during resurgence, or for that matter adaptation, remain largely unexplored. Intriguingly, CT fibers were recently shown to exhibit a marked resistance to use-dependent inactivation and a relatively stable latency during repetitive stimulation. Whether this peculiar conduct of CTs (relative to other C-fiber types) translates into a definable functional characteristic is yet to be elucidated. Nonetheless, the findings of this paper, in addition to our earlier psychophysical observations and other microneurography studies (discussed above), suggest that the responsiveness of CT fibers need not be limited to slowly moving brushing stimuli and nor necessarily follow an inverted U-shaped pattern of neural discharge (in relation to brushing velocity) – features purported as the key to their identification.
High-frequency vibration of the kind used in this study, 200 Hz-200 μm, when applied to the hairy skin of the leg will activate mechanoreceptors located in the skin, the underlying muscle and in the vicinity of joints and interosseous membrane.\textsuperscript{14,37,54} Furthermore, large and small muscle afferents have been implicated in muscle soreness following eccentric exercise.\textsuperscript{60,65} However, in our study, allodynia was cutaneous in origin given that a small amount of intradermal anesthetic, injected around the vibration probe, abolished the vibration-evoked pain. These results may well reflect different stimulus parameters/experimental paradigms. However, it is unclear whether this sensory-perceptual realignment can be treated as a function of the magnitude of muscle damage. Furthermore, little is known about the effect of DOMS on the descending inhibitory system. For example, Nasu and colleagues\textsuperscript{44} proposed that, in the context of repeated cold stress, it is the intensity of noxious stimulation that determines whether the sensory changes remain limited to the muscle, i.e. muscle allodynia/hyperalgesia, or alter the central processing of cutaneous inputs as well. Whether a similar mechanism can explain cutaneous allodynia after eccentric contractions warrants further exploration. Nonetheless, in the context of acute muscle pain, we have shown that cutaneous allodynia need not be limited to an overlap in spinal projections from skin and muscle compartments (see Paper II of this thesis).\textsuperscript{42}

In our experimental paradigm, we can reversibly produce allodynia that complements recent animal work showing activation of superficial dorsal horn neurons by CT-mediated inputs.\textsuperscript{1,30,55} Furthermore, CT-fiber endings are densely concentrated in the substantia gelatinosa, which has been proposed as an integrator of small-fiber inputs from cutaneous and sub-cutaneous sources.\textsuperscript{32,58,59} Converging evidence connotes a
plausible pathway linking CT fibers to central processing wherein inputs project from spinal lamina I neurons\(^1\) via the posterior portion of the ventromedial thalamic nucleus (VMpo)\(^11\) to a network of brain regions including the dorsal posterior insula (dpINS).\(^5,20\) Intriguingly, the pattern of insular activation induced by CT-fiber inputs is near identical to that observed during cutaneous and muscle pain.\(^{25,26}\) The lamina I-VMpo-dpINS system provides a possible mechanism of CT-mediated allodynia in our paradigm.

Recent neuroimaging studies have shown a differential cortical representation of sensory and affective aspects of pain. The activation of the discriminative-cognitive areas, in particular the primary somatosensory cortex, has been associated with the sensory aspect of pain.\(^{27}\) Conversely, the limbic-emotional regions (insula, orbitofrontal and anterior cingulate cortices) have been implicated in the affective dimension of pain.\(^{51}\) Olausson and colleagues\(^{49}\) showed that the somatosensory cortices are not activated by gentle brushing in large-fiber deafferented patients. However, this observation should be treated with some caution given the finding on extensive thinning of the cortex in these patients (personal communication; see also\(^7\)). Clinical studies have shown that insular lesions can result in pain asymbolia,\(^17\) a condition characterised by a non-appreciation of the aversive nature of a noxious stimulus presumably due to the loss of connections between the sensory and affective systems.\(^3\) The integrity of such connections seems vital for the elicitation of an affective/motivational quale of perceptual experience.\(^{22}\) Intriguingly, a unilateral insular lesion can produce a generalized (bilateral) impairment in the emotional responses to pain (the sensory aspect remains intact).\(^3\) Whether the inability of low threshold C fibers to elicit perceptual responses, in the absence of pain, can be linked
to differential activation of affective/limbic vis-à-vis sensory system remains to be ascertained.

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**Disclosures**

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References


33. Löken LS, A pathway for pleasant touch: linking peripheral receptors to central processing and hedonic experience, in Department of Neuroscience and Rehabilitation and Department of Physiology. 2009, University of Gothenburg: Gothenburg.


31


66. Wiklund Fernström K, Physiological properties of unmyelinated low-threshold tactile (CT) afferents in the human hairy skin, in Department of Physiology. 2004, University of Gothenburg: Gothenburg.


PAPER IV
DO C-TACTILE FIBRES HAVE A ROLE IN GENERATION OF MECHANICAL ALLODYNA DURING CUTANEOUS PAIN?

Saad S Nagi & David A Mahns

Abstract
We recently showed that C-tactile (CT) fibres in human hairy skin (anterior leg) can contribute to the crossover between touch and pain, i.e. allodynia. In this case, the sensory crossover was observed during underlying muscle pain. Whether CTs play a similar role when pain originates in the skin remains untested. Detailed psychophysical observations were carried out in 28 healthy subjects. Cutaneous pain was induced by infusing hypertonic saline (5%) into the hairy skin overlying tibialis anterior muscle and an innocuous tactile stimulus (sinusoidal vibration: 200 Hz-200 µm) was applied to the hairy skin ~90 mm distal to the infusion site. Prior to the induction and upon cessation of cutaneous pain, vibration was reported as non-painful. In contrast, during cutaneous pain vibration evoked a significant and reproducible increase in pain (allodynia). The contribution of different fibre classes to allodynia was determined by employing conduction blocks of myelinated (sciatic nerve compression) and unmyelinated (intradermal anaesthesia) fibres. Allodynia persisted during blockade of myelinated fibres but was abolished by blockade of unmyelinated cutaneous fibres. Based on these and our earlier findings, we demonstrate that the allodynic effect of CT-fibre activation is not limited to interactions between superficial (skin) and deep (muscle) compartments but can be equally realised when the tactile and pain-inducing stimuli are confined to a single compartment. These results suggest that CTs may have a broader role in peripheral and central integration of tactile and nociceptive inputs.
Introduction

It is widely appreciated that tactile and pain sensations rely on limited classes of peripheral afferent nerve fibres and specialised receptors. However, it is unclear whether these distinct sensations result from the activation of a single class of afferent fibre or the convergence of inputs from multiple classes. Resolving this lack of clarity may be clinically advantageous, particularly in conditions where the distinction between touch and pain is blurred such that an otherwise innocuous tactile stimulus is perceived as painful, i.e. *allodynia* (Merskey & Bogduk, 1994). Furthermore, in many pain-states, it is not possible to resolve whether the observed allodynia reflects an alteration in the peripheral responsiveness of nociceptive afferents or a perturbed central integration of nociceptive and tactile inputs.

The generation of allodynia during experimentally induced pain as well as pathological pain-states is often linked to the activation of low-threshold large-diameter (Aβ) mechanoreceptors (Campbell *et al.*, 1988; Treede & Cole, 1993; Wasner *et al.*, 1999; Maihöfner *et al.*, 2003). An attribution reinforced by observations that electrical intraneural microstimulation of large mechanosensitive units in the context of pain evoked allodynia, whereas the use of large-fibre blocks (compression or ischaemic) abolished this effect (Gracely *et al.*, 1992; Torebjörk *et al.*, 1992; Cervero & Laird, 1996). In contrast, the use of similar techniques in other studies involving patients with pain-producing conditions have argued for a role of small-diameter ‘nociceptive’ afferents (Cline *et al.*, 1989; Price *et al.*, 1992). What lent credence to this finding was the abolition of alldyinia following blockade of small-diameter presumed nociceptors in patients with ongoing pain (Arner *et al.*,...

The existence of C low-threshold mechanoreceptors (CLTMRs) was documented long ago in the hairy skin of non-human primates and sub-primates (Zotterman, 1939; Maruhashi \textit{et al.}, 1952; Douglas & Ritchie, 1957; Bessou \textit{et al.}, 1971). Although initially regarded as an evolutionary vestige (Kumazawa & Perl, 1977), recent studies have reported a class of unmyelinated fibres in humans, dubbed C-tactile (CT) fibres, that responds most notably to slowly moving, low-force, mechanical stimuli such as finger stroking and soft brushing (Johansson \textit{et al.}, 1988; Nordin, 1990; Vallbo \textit{et al.}, 1993; Löken \textit{et al.}, 2009). It is unclear whether the responsiveness of CLTMRs can be readily extrapolated to the responsiveness of the CTs in healthy human subjects. Indeed, our understanding of the functionality of CTs is based primarily on observations in two patients with a total dysfunction of large-diameter fibres and at least a partial impairment of small-diameter fibres (sensory neuronopathy syndrome: Sterman \textit{et al.}, 1980; Olausson \textit{et al.}, 2002; Olausson \textit{et al.}, 2008). In these patients, the perceptual responses to light mechanical stimuli varied from “no sensations at all” to “a very weak, vague, and inconsistent” sensation of light touch that was “slightly or moderately pleasant” (Olausson \textit{et al.}, 2010). In normal subjects, parallel shifts in CT-fibre discharge and affective rating evoked by brushing stimuli were construed as a link between CTs and pleasantness (Löken \textit{et al.}, 2009). However, it should be noted that brush stroking can also evoke a neutral or unpleasant sensation at the lowest brushing velocities, suggesting that gentle tactile stimulation can elicit positive and negative affective dimensions of touch (Löken \textit{et al.}, 2009; Löken \textit{et al.}, 2011). A role for CTs in pain processing in healthy
human subjects was identified in our recent work (Nagi et al., 2011) by applying noxious (hypertonic saline) and tactile (vibration and brushing) stimuli to the tibialis anterior muscle and the overlying skin respectively. It was demonstrated that mechanosensitive C fibres, not necessarily of a nociceptor type, in the skin can contribute to touch-evoked allodynia. Whether the same applies when noxious (hypertonic saline) and tactile (vibration) stimuli are applied to the skin remains untested in conscious human subjects (Jänig, 2011).

**Methods**

In order to test whether our earlier observations of CT-mediated allodynia during muscle pain (Nagi et al. 2011) are reproducible in a cutaneous pain-state, innocuous tactile (200 Hz-200 µm) and painful (intradermal infusion of 5% hypertonic saline) stimuli were applied to adjacent regions of skin separated by ~90 mm. A total of 28 healthy human subjects (15 males and 13 females) aged 18-40 years, with no reported musculoskeletal disorders, took part in this study. Informed consent was obtained from each subject. All experiments were approved by the UWS Human Research Ethics Committee (approval number: H9190) and complied with the principles of the Declaration of Helsinki.

Each experiment examined the impact of cutaneous vibration (200 Hz-200 µm) on cutaneous pain induced by injecting hypertonic saline (HS: 5%) into an adjacent region of hairy skin overlying the tibialis anterior (TA) muscle. In all experiments, subjects sat comfortably on a chair with both legs resting on a bench top. As TA is readily palpable during inversion of the foot and dorsiflexion of the ankle joint (Drake et al., 2005), the subjects were asked to perform these movements in order to
identify the anatomical boundaries of TA and to exclude any unappreciated or undisclosed tenderness.

Cutaneous vibration

A circular Perspex (Plexiglas) probe with a rounded 4 mm diameter tip was gently placed against the skin overlying TA without compressing the underlying structures. The probe was positioned normal to the skin surface, ~150 mm distal to the tibial tuberosity and ~15 mm lateral to the anterior border of tibia. The probe was attached to a feedback-controlled sinusoidal stimulator similar to that used in earlier studies (Mahns et al., 2006; Nagi et al., 2011). Vibration was applied prior to and following induction of cutaneous pain. Vibration lasted 30 s and was repeated at 45 s intervals. This was aimed at providing sufficient time between trials in order to circumvent desensitization in activated mechanoreceptors – large-diameter as well as small-diameter (Iggo, 1960; Wiklund Fernström, 2004; Sahai et al., 2006). The frequency (200 Hz) and amplitude (200 µm) values were carefully chosen as the resulting stimulus is clearly innocuous (Talbot et al., 1968; Merzenich & Harrington, 1969; Mahns et al., 2006) but also based on our previous findings that it can evoke CT-mediated allodynia when applied during muscle pain (Nagi et al., 2011).

Hypertonic saline-induced cutaneous pain

To induce cutaneous pain, a 30G needle was inserted just beneath the skin surface, then advanced (5-10 mm) parallel to the skin surface and held in place using microporous adhesive tape: the tip of the cannula was positioned ~60 mm distal to the tibial tuberosity and ~30 mm lateral to the anterior border of tibia. The needle was connected via scalp vein set (non-toxic, non-pyrogenic) to a computer-controlled
syringe pump (model 55-2226, Harvard Apparatus, Holliston, MA, USA). The rate of HS-infusion was initially set at 50 µl min\(^{-1}\) and the response of the subject closely monitored. In control experiments, which were conducted in separate sessions, 5 subjects were asked to indicate any perceptual consequences during infusion of normal saline at room temperature (0.9%; infusion rate: 50 µl min\(^{-1}\); 2 min duration; tested in duplicate at 2 min interval) on a pain scale (Wolff & Jarvik, 1963). In test experiments, once the subject identified the onset of the pain associated with the infusion of HS, which typically came about in ~15-30 s, the infusion rate was adjusted (where needed) in order to maintain a constant pain rating. Once a stable baseline pain was obtained, no further adjustments were made to the infusion rate for the duration of the experiment.

Subjects rated the perceived pain intensity using a rotating dial mounted on a Visual Analogue Scale (VAS). The VAS was divided into ten equal segments within a range of 0 (no pain) to 10 (intolerably intense pain) over 300° of rotation. Pain intensity reported by the subjects was recorded on a computer using a digital-to-analogue converter and interface software (Spike2 version 6.05a, Cambridge Electronic Design, Cambridge, England). Using the VAS dial, subjects provided a continuous rating of pain prior to, during and upon cessation of HS-infusion into the skin. Once cutaneous pain was initiated and had remained steady for at least 1 min, the intermittent (30 s on, 45 s off) 200 Hz-200 µm vibration was re-commenced. At least two consistent (and consecutive) responses to vibration were required before progressing with the experiment. In order to avoid response/expectation bias, subjects were instructed that the HS-induced pain could remain the same, increase or decrease during vibration. White noise was delivered through headphones to ensure
that auditory cues associated with mechanical stimulator were not detected by the subjects. Furthermore, this approach is consistent with other psychophysical studies that reported an enhanced vibrotactile sensibility when subjects were tested in presence of white noise (Merzenich & Harrington, 1969).

**Series 1. Cutaneous local anaesthesia**

VAS responses to at least two consecutive vibration trains were recorded in 19 subjects. Thenceforth, in 12 of those subjects, 0.2-0.4 ml of a local anaesthetic (Xylocaine: 0.25%) was injected into the skin region stimulated by vibration in order to preferentially block the C-fibre inputs. The effectiveness of the C-fibre block was ascertained by the loss of perceptiveness to warm stimuli (~40°C, brass rod in contact with the skin for 5 s), whereas the preservation of vibration (20 & 200 Hz; 200 µm; 30 s duration) and cool (~15°C, brass rod in contact with the skin for 5 s) sensations were taken for the intactness of myelinated fibres (Torebjörk & Hallin, 1973; Mackenzie et al., 1975; Hallin & Torebjörk, 1976). Vibration was applied within the anaesthetized skin (C fibres absent) and in the adjacent non-anaesthetized skin (all fibres intact).

**Series 2. Compression block of sciatic nerve**

In 14 subjects (5 naive), a compression block of sciatic nerve was used with the aim of knocking out the myelinated fibres, thereby examining the contribution of C fibres alone to allodynia. However, a clear blockade could not be achieved in 3 subjects, hence they were excluded (data not shown). A metal bar was placed just distal to ischial tuberosity to apply compression to sciatic nerve. Induction of HS-induced cutaneous pain was timed to coincide with the preferential blockade of large- and
small-diameter myelinated afferents. The blockade of myelinated fibres was confirmed by the loss of vibration (20 & 200 Hz; 200 μm) and cool (~15°C brass rod) sensibility. Continuity of the C-fibre inputs was confirmed by the preservation of warm (~40°C brass rod) sensibility. In addition, the production of cutaneous pain (HS-induced) within the skin region of absent myelinated fibres was itself an indication of intact C fibres (Mackenzie et al., 1975; Ochoa & Torebjörk, 1989; Weerakkody et al., 2003; Nagi et al., 2011). Somatosensory sensibility within the skin region affected by compression block was compared with regions on the medial aspect of the experimental leg (innervated by femoral nerve: Drake et al., 2005), and the contralateral leg. VAS responses to vibration were recorded in the skin region with blocked myelinated fibres. In addition, akin to Series 1, a small amount of low-dose anaesthetic (Xylocaine 0.25%) was injected around the vibration site in order to block C fibres – the only class of fibres intact within the innervation territory of sciatic nerve during compression block. The effectiveness of the C-fibre block was ascertained by the loss of warm sensibility. Vibration was applied within the anaesthetized region (all cutaneous fibres blocked) as well as in the adjacent non-anaesthetized skin (C fibres intact).

Statistical analysis

Each VAS response to vibration was compared to the baseline HS-induced cutaneous pain (base) observed just prior to vibrotactile stimulation and treated as an independent, sequential event. The responses to vibration were also expressed as a percentage of the baseline cutaneous pain. A one-way repeated measures analysis of variance (ANOVA) was used to determine if there was a significant difference between any of the groups (Zar, 1984). Where a significant difference was indicated
(\(P < 0.05\)), specific groups were compared using a Newman-Keuls multiple comparison test. All statistical comparisons were completed using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). All data are presented as mean ± standard error of the mean (SEM).

**Results**

In 21 of the 25 subjects where HS was infused, a stable baseline was obtained that persisted for the duration of the experiment. Data from the remaining 4 subjects were discarded because a steady background pain could not be achieved or a vasovagal response presumably elicited during HS-infusion (van Lieshout et al., 1991). HS was not used in those 3 subjects where compression block failed to take effect. In parallel experiments (\(n = 5\)), the infusion of normal (0.9%) saline into the skin was reported as non-painful (VAS = 0). Although correlations between the magnitude of HS-induced pain and vibration-evoked responses are shown in Fig. 1a, this was not the primary focus of this study. Instead, the aim was to identify the peripheral origin of vibration-evoked allodynia during HS-induced cutaneous pain. Prior to the induction of cutaneous pain, all subjects reported that the application of vibration (200 Hz-200 \(\mu\)m; 30 s duration; *see* Methods) to the skin overlying TA was non-painful. Vibratory sensations were described as a combination of localized pressure at the vibration site, and diffuse vibration within the leg.

*Vibration-evoked allodynia*

Following the onset of HS-induced cutaneous pain, a reproducible \((n \geq 3)\) vibration-evoked increase (*allodynia*) in the overall pain rating was observed on a trial-by-trial basis in all 19 subjects (Fig. 1a).
Figure 1. Reproducible effect of vibration on hypertonic-saline induced cutaneous pain.
Duplicate vibration-evoked responses of each subject (a) and the mean data (b) during cutaneous pain are shown (±SEM; n = 19; grey: allodynia; unshaded: baseline cutaneous pain). Vibration evoked a reproducible increase in baseline pain across all subjects (triangular points, above the line of unity). This effect was significant. *** $P < 0.001$
Consistent with the individual responses, the mean data (±SEM; Fig. 1b) revealed a significant (ANOVA: $F = 47.47, P < 0.0001$) and reproducible increase in the intensity of HS-induced cutaneous pain (base: 3.8 ± 0.3, 3.8 ± 0.3) during vibration (vibration: 4.9 ± 0.3, 4.9 ± 0.3; $P < 0.001; n = 19$). Furthermore, consistent with the stability of HS-induced pain and the reproducibility of vibration-evoked allodynia, there were no significant differences ($P > 0.05$) between duplicate base or vibration values.

**Series 1. Allodynia abolished by blockade of cutaneous C fibres**

Low-dose intradermal anaesthetic (0.25% Xylocaine) was used to block the action of cutaneous C fibres, while the myelinated fibres remained intact, in order to examine the effect of this selective blockade on the generation of allodynia. The loss of C-fibre activity was confirmed by the abolition of warm sensibility and the preservation of vibration and cool sensations. As shown in Fig. 2, prior to intradermal anaesthesia, HS-infusion evoked a steady baseline pain (base: 100%) that increased significantly during vibration (vibration: 129.2 ± 4.8%, 129.4 ± 4.6%; $P < 0.001; n = 12$). No significant differences ($P > 0.05$) between duplicate HS-induced pain (base) or between duplicate vibration-evoked responses were observed. Following C-fibre cutaneous block, the vibration-evoked responses were statistically indistinguishable from the baseline HS-induced pain (base: 100%; vibration: 110.0 ± 4.4%, 111.9 ± 5.1%; $P > 0.05; n = 12$). In other words, the allodynia evoked within the anaesthetized region was significantly reduced ($P < 0.001$) relative to the vibration-evoked responses prior to the anaesthesia of C fibres.
Figure 2. Alldynia abolished by conduction block of cutaneous C fibres. Mean vibration-evoked responses (±SEM; n = 12; grey) were expressed as a percentage of the baseline (HS-induced) cutaneous pain. Prior to intradermal anaesthesia, vibration evoked a significant increase in baseline cutaneous pain (alldynia). Within the anaesthetized skin (C fibres blocked), the alldynic effect of vibration was markedly attenuated and statistically indistinguishable from the baseline. In the adjacent non-anaesthetized skin with all fibres intact, alldynia was preserved. *** P < 0.001
The attenuation/abolition of allodynia in the anaesthetized skin was apparently not due to a decline in the capacity of peripheral nerve fibres to mediate this effect and nor an inability of the central nervous system to integrate/process the information, as the vibration-evoked allodynia was preserved in the adjacent non-anaesthetized skin (base: 100%; 124.0 ± 4.7%, 125.0 ± 4.9%; \( P < 0.001; n = 12 \)). Given that allodynia was reproducibly evoked while all fibres were intact, but failed to elicit from the anaesthetized skin, signifies the role of cutaneous C fibres in mediating this effect (ANOVA: \( F = 18.88, P < 0.0001 \)).

**Series 2. Allodynia persists during blockade of myelinated fibres**

A compression block of the sciatic nerve was employed in order to thwart the action of myelinated fibres and assess the role of the residual (C-fibre) input in mediating allodynia. The preferential blockade of myelinated fibres was confirmed by the abolition the vibration and cool sensations, whereas the intactness of C fibres was confirmed by the preservation of HS-induced pain and, in particular, warm sensibility. In contrast to the selective C-fibre block, where allodynia was significantly attenuated, vibration-evoked allodynia was preserved following the blockade of myelinated fibres (base: 100%; vibration: 127.4 ± 6.0%, 127.8 ± 7.4%; \( P < 0.001; n = 11 \)); an effect that was abolished, as shown in Fig. 3, by subsequent blockade (0.25% Xylocaine) of the residual cutaneous (C-fibre) input (base: 100%; vibration: 103.7 ± 2.5%, 103.7 ± 2.5%, \( n = 11; P > 0.05 \)). The abolition of allodynia was not due a generalized decline in the responsiveness of peripheral or central neurons as the effect was preserved in the adjacent non-anaesthetized skin (base: 100%; 130 ± 7.3%, 131.5 ± 8.6%; \( P < 0.001; n = 11 \)).
Figure 3. **Alloodynia persisted during conduction block of myelinated fibres.** Mean vibration-evoked responses (±SEM; n = 11; grey) were expressed as a percentage of the baseline (HS-induced) cutaneous pain. Vibration evoked a significant increase in baseline cutaneous pain (alloodynia) regardless of the blockade of myelinated fibres. In contrast, allodynia was abolished by the blockade of residual (C-fibre) input from the skin region with absent myelinated fibres. In the adjacent (non-anaesthetized) skin with C fibres intact, the allodynic effect of vibration was preserved. *** $P < 0.001$
Given that allodynia elicited regardless of the blockade of myelinated fibres, but was abolished in the absence of cutaneous C fibres, attests to the capacity of the latter to modulate cutaneous pain (ANOVA: $F = 12.50, P < 0.0001$).

**Discussion**

The aim of this study was to investigate the role of low-threshold C fibres in tactile-modulation of cutaneous pain. In these experiments, we used a slow infusion (50 µl min$^{-1}$) of hypertonic saline to produce localized pain in the skin. In contrast, the infusion of normal saline (50 µl min$^{-1}$) in parallel experiments (predictably) failed to evoke any discernible sensation (*see* Wolff & Jarvik, 1963; Graven-Nielsen, 2006).

Prior to the induction of cutaneous pain, all the subjects described vibration as non-painful. However, following induction of cutaneous pain, a reproducible increase in the pain intensity was reported by previously innocuous vibration. This allodynic response was preserved following blockade of myelinated fibres, but was abolished following blockade of cutaneous C fibres (while the myelinated fibres were conducting or not). Consequently, we now provide evidence that our earlier observations (*Nagi et al.*, 2011), wherein CTs were shown to mediate allodynia during deep (muscle) noxious stimulation, are not limited to cross-compartment interactions but can be evoked within adjacent regions of skin.

Some of the earlier work engenders a potential ambiguity, whether allodynia is a product of a central change in the integration of sensory inputs or a peripheral change in primary afferent fibre responsiveness. This potential ambiguity is largely due to the confinement of both innocuous and noxious stimuli to a single anatomical compartment. We avoided this ambiguity previously by working across two
compartments, i.e. applying a noxious stimulus to one compartment (muscle) and a tactile stimulus to a separate compartment (skin). In our earlier study (Nagi et al., 2011), we argued that, in a two-compartment model, any change in the responsiveness, or sensitization, of cutaneous afferent fibres following intramuscular injection of hypertonic saline was highly unlikely. On this occasion, we likewise argue that peripheral sensitization is unlikely to account for the observed allodynia given the separation (~90 mm) between the stimulation sites for vibration and HS-infusion. Furthermore, the spread of HS to the distal site (where vibration was applied) seems unlikely considering the slow infusion rate (50 µl min⁻¹), the localized nature of pain and the rapid abatement of pain following cessation of infusion. At the infusion rates used in this study, the pain did not appear to be due to the localized distension at the infusion site given that no sensation, most notably the absence of pain (VAS = 0), was reported when normal saline (0.9%) was substituted for HS (5%).

In both HS-induced responses reported here and in our earlier work, the reproducible increase in pain intensity during vibration appears to be more consistent with altered responsiveness (sensitization) at the spinal (dorsal horn) or supraspinal level of the nervous system. Thus, sensitization of dorsal horn neurons may result in a shift in their response properties such that those neurons previously insensitive to a given afferent input may become responsive due to a fall in thresholds or an increase in responsiveness or a combination of both (Woolf, 1983; Woolf & Thompson, 1991; Woolf & Salter, 2000). Thus, central sensitization may manifest as allodynia which involves an integration of pain and tactile modalities (Merskey & Bogduk, 1994). This is consistent with electrophysiological and molecular-genetic studies showing
that CT-fibre endings are densely concentrated in the superficial laminae of dorsal horn (Seal et al., 2009; Andrew, 2010; Li et al., 2011). Indeed, central sensitization may well explain the context-specific contribution of CTs to perception, namely, the lack of a distinct percept under normal conditions and the production of pain during background nociceptive input. It is tempting to suggest that a better understanding of the mechanisms that underlie these contextual boundaries may allow for the possibility of modulating the ‘qualia’ of pain.

As outlined in the Introduction, large-fibre conduction blocks (compression or ischemic) have been used to argue for (Gracely et al., 1992; Torebjörk et al., 1992; Koltzenburg et al., 1994; Cervero & Laird, 1996) and against (Cline et al., 1989; Price et al., 1992) the participation of large-diameter fibres in allodynia. Indeed, the result presented in this paper need not preclude the involvement of large-diameter fibres in view of the following: when all fibres were intact, the allodynia was greatly attenuated, albeit not entirely abolished, following C-fibre blockade (Fig. 2); during compression block of myelinated fibres, the allodynia was entirely abolished following C-fibre blockade (Fig. 3). This suggests that both myelinated and CT fibres can contribute to allodynia and that the relative contribution of each class may well be stimulus- and context-dependent. Indeed, the seemingly unequivocal demonstration of large fibre-mediated allodynia – generated by intraneural microstimulation of large-diameter fibres in a capsaicin model of experimental pain – is subject to complex (and varied) temporal and spatial patterns (Torebjörk et al., 1992). Most notable was the apparent necessity for there to be an overlap between the region of secondary hyperalgesia (evoked by intradermal capsaicin) and the site at which intraneural stimulation evoked a percept of touch in order for there to be a
perceptual shift from innocuous to noxious sensation during activation of large-diameter fibres. In contrast, the CT-mediated effect presented in this paper exhibits a much less constrained interaction such that pain at one site rendered tactile stimulation distally as painful. This broader participation of CTs in the central integration of tactile and nociceptive inputs was supported by our observations in cross-compartment models within the leg (Nagi et al., 2011) and forearm (see Paper II of this thesis). In case of the latter, when muscle pain was induced in a forearm muscle (flexor carpi ulnaris), the allodynia was not only evoked in the hairy skin overlying the forearm but extended to the glabrous skin of the little and index fingers innervated by the ulnar and median nerves respectively. Hence, converging evidence connotes a ubiquitous representation of CT-mediated allodynia in the context of cutaneous and deep somatic pain. Furthermore, this is in conformity with certain pathological conditions wherein the spread of pain does not necessarily follow an orderly somatotopic pattern (extraterritorial pain: Sang et al., 1996; Baron, 2009).
References


