C fibres facilitate proprioceptive afferent transmission to motoneurons

by

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ABSTRACT

Activation of α5 $GABA_A$ receptors located on nodes of primary afferents have been found to produce a long lasting tonic primary afferent depolarization in the spinal cord (tonic PAD). In particular, C fibres have been found to be especially effective in eliciting this tonic PAD. Here by activating C fibres either chemically (via icilin) or electrically (at 50 x T, threshold), we were able to investigate the effects of this C fibre induced tonic PAD on sensory transmission to motoneurons by measuring monosynaptic reflexes (MSRs), extracellular field potentials from afferents and spike transmission in afferents themselves (spikes evoked by PAD: dorsal root reflexes; DRRs). We also used the $5HT_{1D}$ receptor agonist, zolmitriptan, to inactivate C fibres because these 5HT receptors are found exclusively on C fibres. Consequently, we also investigated the effects of inactivating C fibres on sensory transmission. We found that C fibre induced tonic PAD facilitated spike transmission within the spinal cord resulting in increased MSRs, extracellular fields and DRRs. As we expected, when we inactivated C fibres using zolmitriptan, this reduced spike transmission resulting in decreased MSRs, extracellular fields and DRRs. These findings demonstrate the effects C fibres have on sensory transmission via their ability to elicit a long lasting tonic PAD. We also show that C fibres make direct contacts with GABAergic neurons, demonstrating how C fibres can indirectly produce tonic PAD.

PREFACE

Chapter 2 of this thesis will be submitted to Journal of Neurophsiology with the authors: Sophie Black, Yaqing Li, Shihao Lin, Ana M. Lucas-Osma, Krishnapriya Hari, Marilee Stephens and David J. Bennett. I designed and participated in all electrophysiology experiments, interpreted the results and wrote the paper, under the supervision of Dr David J. Bennett. Yaqing Li, Shihao Lin and Krishnapriya Hari assisted with electrophysiology experiments, Ana M. Lucas-Osma completed the histology and microscopy analysis and Yaqing Li and Marilee Stephens assisted with data analysis.

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Background on primary afferent depolarization (PAD)

The spinal cord receives extensive innervation from the periphery via primary afferents which is gated by central intraspinal mechanisms to limit and direct sensory transmission. A fundamental mechanism for controlling afferent neurotransmission is via prior depolarization of the afferents, termed primary afferent depolarization (PAD), which modulates sensory transmission (Eccles et al. 1962; Rudomin and Schmidt. 1999; Wall. 1958).

PAD occurs as a result of GABAergic interneurons directly innervating the axons of sensory afferents that modulate sensory transmission in the spinal cord (Eccles et al. 1962; Rudomin and Schmidt. 1999; Russo et al. 2000). This PAD relies on the unusually high intracellular content of chloride ions in sensory axons which is due to the balance of chloride transporters. There are two cotransporters which are able to transport chloride either into or out of a cell: the $Na^+/K^+/Cl^-$ cotransporter isoform 1 (NKCC1) transporter transports chloride into the cell, whereas the K^+/Cl^- cotransporter isoform 2 (KCC2) extrudes chloride from the cell using energy from the electrochemical gradient of K^+ ions created by the Na^+ K^+ ATPase. Sensory axons are unusual in that they mainly have the NKCC1 cotransporter and mostly lack the KCC2 cotransporter resulting in a high intracellular chloride content (Chamma et al. 2012; Hasbargen et al. 2010). PAD is initiated by the activation of $GABA_A$ receptors which increases the axons permeability to chloride ions, causing an outward movement of chloride ions (due to high intracellular chloride) which then causes a depolarization (PAD). Oddly enough, axons themselves can activate GABA neurons that then produces PAD in afferents. For example, stimulation of low threshold proprioceptive afferents is well known to produce a fairly rapid PAD lasting less than 100 ms, termed phasic PAD (Barron and Mathews. 1939; Eccles et al. 1962; Wall. 1958; Willis. 1999). This classic phasic PAD is thought to be mediated by a trisynaptic loop where

proprioceptive or cutaneous afferents synapse onto an excitatory glutamatergic neuron which, in turn, synapse onto an GABAergic neuron which then synapses back onto the proprioceptive afferent via axo-axonic contacts (Delgado-Lezama et al. 2013; Eccles et al. 1962; Hughes et al. 2005; Rudomin. 2000). The glutamatergic neuron in this trisynaptic loop allows PAD to spread across the midline of the spinal cord, as well as across several spinal segments (Lidierth. 2006; Rudomin. 2000; Lucas-Osma et al. 2018). In contrast, the GABAergic neurons are smaller interneurons (Fink et al. 2014; Hughes et al. 2005).

Classically PAD has been closely associated with presynaptic inhibition of afferents. The concept of presynaptic inhibition of afferent terminals as we understand it today was first developed in the 1950s, but presynaptic inhibition wasn't linked to PAD until Eccles started his research in the 1960s (Eccles et al. 1962; Eccles et al. 1963). Presynaptic inhibition can be defined as inhibition of sensory afferent terminals on motoneurons, which causes less transmitter release from afferent terminals onto these motoneurons, and specifically reduces the monosynaptic reflex (MSR). The concept of presynaptic inhibition was initially proposed by Frank and Fuortes (1957) who observed that following stimulation of group I afferents from flexor muscles, there was a depression of Ia monosynaptic excitatory postsynaptic potentials (EPSPs) without any change in motoneuron excitability. They postulated that this could be due to the postsynaptic changes taking place far out on motoneuron dendrites and therefore could not be detected by their intracellular microelectrode, or alternatively, the conditioning flexor nerve stimulation interacted with the excitatory volley before this excitatory volley arrived at the motoneuron surface, therefore this blocked transmission in the terminals resulting in a reduced EPSP. This is what is termed presynaptic inhibition in the classic literature (Frank and Fuortes. 1957; Rudomin and Schmidt. 1999). However, later Eccles and colleagues linked presynaptic

inhibition to PAD. They ascertained that the extensor motoneuron MSR was profoundly inhibited by a brief flexor nerve conditioning stimulus (Eccles et al. 1962). Further research by Eccles and colleagues (1962) found that group I afferent volleys in the nerves to flexor muscles of the cat hindlimb are very effective in producing a prolonged depolarization of the group I afferents from all types of muscle. They were able to do this by recording intracellularly from muscle afferent fibres in the dorsal columns and also recording the dorsal root potential (DRP; PAD can be measured by following its antidromic electrotonic spread to other dorsal roots as a DRP). They then postulated that this presynaptic depolarization is responsible for the reduced monosynaptic EPSPs that Frank and Fuortes (1957) first observed because its timing was similar to the time course of presynaptic inhibition, though in retrospect this was only a correlational link. It was proposed that this presynaptic depolarization reduced the size of the presynaptic impulse causing decreased release of excitatory transmitter (Eccles et al. 1962) and is modulated by $GABA_A$ receptors, as they found that both PAD and presynaptic inhibition are reduced by the $GABA_A$ antagonists picrotoxin and bicuculline (Eccles et al. 1963). They thought that PAD inhibits transmitter release by shunting or inactivating spikes invading the terminals, or by interfering with calcium currents that release transmitter (Bardoni et al. 2013; Rudomin and Schmidt. 1999; Stuart and Redman. 1992). So following several decades of research, it was thought that many afferents activate a trisynaptic loop, which activates $GABA_A$ receptors on the proprioceptive afferent terminal boutons, leading to a depolarization of these afferents and ultimately causing presynaptic inhibition of the MSR (Frank and Fuortes. 1957; Eccles et al. 1962), as detailed above.

PAD's excitatory actions

More current research also demonstrates that phasic PAD and GABA can have excitatory actions (Bos et al. 2011; Willis. 1999; Lucas-Osma et al. 2018) and in some cases PAD evoked by a dorsal root stimulation can be so large that it directly excites afferents and produces spikes in proprioceptive afferents (Eccles et al. 1961; Lucas-Osma et al. 2018; Rudomin and Schmidt. 1999). Theses spikes travel antidromically along afferents and are termed dorsal root reflexes (DRRs). These DRRs can be measured experimentally by stimulating a dorsal root (or nerve) and recording the antidromic spikes propagating out an adjacent dorsal root. These spikes evoked by PAD (DRRs) have been found to not only propagate distally, but also to release transmitter centrally to evoke excitatory postsynaptic potentials (EPSPs) in motoneurons, thus contributing to larger more widely spread reflexes (Eccles et al. 1961; Willis. 1999). Interestingly, DRRs have been associated with neurogenic inflammation and arthritis (Willis. 1999). It has been shown that antidromic electrical stimulation of afferent fibres can cause neurogenic inflammation (Ferrell and Russell 1986; Szolcsányi. 1988). It has been proposed that tissue inflammation or injury causes excessive PAD to occur causing enhanced central release of GABA, resulting in DRRs travelling antidromically from the spinal cord to the injury site (Sluka et al. 1995). Neuropeptides (particularly substance P and calcitonin gene-related peptide; CGRP) found in peripheral terminals of nociceptive fibres are then released causing this neurogenic inflammation (Lobanov et al. 2011). It has been shown that DRRs are particularly prevalent in acute arthritis and these DRRs are in part conveyed by capsaicin sensitive afferents, presumably C fibres or possibly A delta fibres (Rees et al. 1996; Willis. 1999). It has been proposed that during inflammation, DRRs in *A*β fibres activate GABAergic inhibitory interneurons which consequently activate DRRs in C fibres (Cervero and Laird. 1996). These activated C fibres then cause pain; this is a possible mechanism for allodynia (Cervero and Laird. 1996). DRRs as a result of PAD is an

interesting concept as it superimposes an excitatory mechanism (DRRs), onto an inhibitory mechanism (presynaptic inhibition due to PAD as outlined earlier). Instead of the classical inhibition caused by PAD, debilitating conditions such as allodynia can occur from the central effects of DRRs, and other conditions such as inflammation and arthritis may occur from the peripheral effects of DRRs (Willis. 1999). This demonstrates the importance of DRRs in certain medical pathologies. We wanted to investigate here how C fibre activation and inactivation effects these DRRs. Our findings could potentially shed some insight on future drug treatments for the medical conditions discussed previously.

Latest research on PAD and the emergence of a long lasting tonic PAD

Unlike the classic ideas of PAD and presynaptic inhibition outlined previously, recent research has shown that $GABA_A$ receptors are not found on most proprioceptive afferent terminals contacting motoneurons (Lucas-Osma et al. 2018), throwing into doubt the classic concept of terminal presynaptic inhibition by terminal $GABA_A$ receptors. Interestingly, $GABA_A$ receptors that produce PAD were found to be densely located at nodes near branch points in large myelinated afferents, specifically the α5 $GABA_A$ receptor subtype (Lucas-Osma et al. 2018). The GABA-mediated PAD described above is generated at these nodes where α5 $GABA_A$ receptors are found and not at terminals (Lucas-Osma et al. 2018).

We have found that these extrasynaptic α5 $GABA_A$ receptors generate a spontaneous tonic depolarization of afferents (tonic PAD) which facilitates spike transmission (Lucas-Osma et al. 2018). A long lasting tonic PAD like this has been suggested previously (Delgado-Lezama et al. 2013). This tonic PAD increases with repeated dorsal root stimulation and even persists after

synaptic transmission is blocked, suggesting that is it caused by GABA leak or spillover (Lucas-Osma et al. 2018). As already mentioned, these α5 $GABA_A$ receptors are located at branch point nodes far from afferent terminals directly mirroring the location of Na^+ channels. It is at these branch points where, theoretically, propagating action potentials are most vulnerable to failure. This confirms the work started by Walmsley and colleagues who observed these nodal boutons (or as they refer to them en passant boutons) that have synaptic specializations that have GABA input (Walmsley et al. 1995). Indeed, we found previously that while dorsal root evoked phasic PAD initiates some antidromic spikes in Ia proprioceptive afferents (DRRs), increasing tonic PAD facilitates these antidromic spikes, causing less failure of these spikes to propagate into the afferent branches. Furthermore, blocking tonic PAD has the opposite effect (Lucas-Osma et al. 2018). Likely, tonic PAD and the associated α5 $GABA_A$ receptors generally facilitate spike transmission because they bring failed spikes closer to sodium threshold at these branch points where spike failure is likely to occur (Lucas-Osma et al. 2018). We found that stimulation of all sizes of sensory afferents are able to elicit this long lasting tonic PAD, but interestingly C fibres were particularly effective in producing a large and long lasting tonic PAD (Lucas-Osma et al. 2018). Even when all central spike transmission was blocked using low dose tetrodotoxin (TTX), C fibre stimulation still evoked a tonic PAD, presumably because some C fibres are TTX resistant (Lucas-Osma et al. 2018). It is this C fibre evoked PAD this thesis focuses on. We wanted to investigate what the contribution of C fibres is in facilitating spike transmission via a tonic PAD. We did this by activating C fibre electrically at 50 x T (where T = afferent threshold) and chemically, via the application of drugs that activate receptors found on C fibres, to be discussed in more detail next. C fibres are a very important component of pain and associated medical conditions (Garland. 2012). As such, understanding their role in producing a tonic PAD

and the consequences this might have on sensory transmission within the spinal cord is important to be able to create effective pharmaceutical treatments for many medical and pain conditions.

Role for $GABA_B$ receptors

We know there is a unique genetically defined population of GABAergic neurons, namely GAD2 neurons that are known to make small axo-axonic synapses with sensory afferent terminals in the ventral horn (Fink et al. 2014). However, as outlined above, $GABA_A$ receptors are not found on afferent terminals. As such, this suggests a role for another GABA receptor acting on afferent terminals to motoneurons, most likely $GABA_B$ receptors. Indeed, $GABA_B$ receptors coupled to Ca^{2+} channels on primary afferent terminals have been found to mediate presynaptic inhibition (Curtis et al. 1981; Curtis et al. 1986).

C fibres and the monosynaptic reflex

We also wanted to investigate how C fibre activation or inactivation effects the monosynaptic reflex (MSR) as this can be an indirect indicator of the amount of afferent spike transmission to motoneurons. Large proprioceptive afferents (mainly group Ia) directly connect to motoneurons, making the MSR, which leads to the stretch reflex. One source of inhibitory control over the MSR is serotonin (5-HT), which is known to inhibit the MSR via the actions of $5\text{-}HT_1$ receptors (Honda et al. 2004; Honda et al. 2006; Lucas-Osma et al. 2019). Following certain injuries to the central nervous system, this brainstem derived 5-HT is either partially or fully eliminated causing exaggerated MSRs which, in part, is due to a loss of this 5-HT derived inhibitory control (Murray et al. 2010; Schmidt and Jordan. 2000). There are a multitude of different 5-HT

receptors within the spinal cord (e.g. 5-HT $_{1A, 1B, 1D, 1F}$) (Amrutkar et al. 2012). Previous work from the lab investigated which of these 5-HT receptor subtypes is involved in regulating the MSR (Lucas Osma et al. 2019). We found that application of the selective 5-HT$_{1D}$ receptor agonist zolmitriptan significantly reduced the MSR, with the MSR recorded on ventral roots being largely eliminated with the application of zolmitriptan, whereas 5-HT agonists without affinity to 5-HT$_{1D}$ receptors had no effect on the MSR. Zolmitriptan also activates 5-HT$_{1B}$ receptors but at a much higher dose than we find inhibits the MSR (Lucas-Osma et al. 2019; Tepper et al. 2002), and indeed zolmitriptan's actions on the MSR is specifically blocked by 5-HT$_{1D}$ antagonists and not 5-HT$_{1B}$ antagonists. Interestingly, 5-HT$_{1D}$ receptors are found exclusively on C fibres (Lucas-Osma at el. 2018; Potrebic et al. 2003). C fibres are unmyelinated, high threshold, polymodal noxious afferent fibres that carry sensory information to Rexed laminae I and II of the spinal cord where they synapse with secondary order neurons (D'Mello and Dickensen. 2008; Potrebic et al. 2003). The axons of these second order neurons cross the midline of the spinal cord and ascend in the ventral and lateral spinothalamic tracts terminating in the thalamus (Garland. 2012). The 5-HT$_{1D}$ receptor is a G protein coupled receptor that activates an intracellular signalling cascade causing inhibition via a reduction in cAMP. This receptor inhibits activity and transmitter release from C fibres (Amrutkar et al. 2012; Zhao et al. 2016) and can be activated by the drug zolmitriptan, as discussed previously. Consequently, zolmitriptan is a useful tool which allowed us to investigate the effects of C fibre inactivation.

Zolmitriptan is one of several triptan drugs that were developed to treat migraines (Potrebic et al. 2003; Tepper and Rapoport. 2002). The 5-HT$_{1D}$ receptors which it activates, are particularly abundant in C fibres within both trigeminal nerve and dorsal root ganglia (Potrebic et al. 2003).

5-HT$_{1D}$ receptors located on the trigeminal nerves receive pain from leptomeningeal vessels and inflammation of these vessels induces both dilation and inflammation of cranial vessels, which is a hallmark associated with migraine (Pascual. 1998). Zolmitriptan is able to cross the blood brain barrier so has both central and peripheral actions on migraine pain. It acts peripherally by inhibiting dilation and inflammation of cranial vessels but it also has a central nociceptive action by interrupting pain signal transmission from these blood vessels to sensory neurons located in the brainstem (Pascual. 1998; Tepper and Rapoport. 2002). Zolmitriptan has proved to be particularly effective in treating migraine because it is highly selective, resulting in reduced or eliminated activity at other receptor subtypes, which can cause unwanted side effects such as vomiting and nausea (Pascual. 1998; Potrebic et al. 2003). We already know that zolmitriptan reduces the MSR (Lucas-Osma et al. 2019), indicating that it increases spike transmission to the ventral horn. Thus, given our previous findings showing that large proprioceptive afferents are able to elicit a tonic PAD which facilitates spike transmission, we wanted to determine the effects of C fibre-induced tonic PAD on sensory transmission, as outlined above. We also wanted to investigate the effects of C fibre inactivation (zolmitriptan) on DRRs, phasic PAD and sensory transmission within the spinal cord. Zolmitriptan is already a well proven effective drug to treat migraine (Potrebic et al. 2003; Tepper and Rapoport. 2002), depending on our findings it could also prove useful to treat particular medical conditions outlined previously.

C fibres and transient receptor potential (TRP) receptors

Our aim in this thesis is to investigate how C fibres affect sensory transmission to motoneurons, as discussed above. C fibres contain a host of TRP receptors which detect a variety of mechanical, thermal and chemical stimuli. Specific drugs can be used to activate these TRP

receptors and thus allows us to selectively activate C fibres (Cortright et al. 2007; Richter. 2019). These TRP receptors are all along the length of C fibres in the spinal cord, as well as in the peripheral tissue they innervate (Anand et al. 2008; Immke and Gavva. 2006). The vanilloid receptors respond to noxious heat (> 43°C) and the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor can be activated by capsaicin (Levine and Alessandri-Haber. 2007; Spicarova et al. 2014). Similarly, the transient receptor potential melastatin 8 (TRPM8) receptor which detects environmental mild cold (cool receptor) and menthol stimuli can be activated by the drug icilin (Bharate and Bharate. 2012; Jankowski et al. 2017). These TRPM8 receptors are activated by cooling to approximately 22°C and are inactivated above approximately 26 - 27°C (Andersson et al. 2004). We will use both capsaicin and icilin to chemically activate C fibres throughout this thesis.

So in summary, in my thesis we intend to investigate the effects on C fibres on afferent transmission within the spinal cord. We hypothesis that activation of C fibres will facilitate proprioceptive afferent transmission via a tonic PAD and inhibiting C fibres will reduce proprioceptive afferent transmission by reducing C fibre induced tonic PAD.

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Chapter 2: C fibres facilitate proprioceptive afferent transmission to motoneurons

A version of this chapter will be submitted to *Journal of Neurophysiology*

INTRODUCTION

The monosynaptic reflex (MSR) is mediated by the activation of proprioceptive afferents that directly synapse onto motoneurons (Imai et al. 2016; Mentis et al. 2011). The MSR is important for the maintenance of balance and posture, forming part of a feedback loop (Gottlieb and Agarwal. 1973; Hill and Vandervoort. 1996). If the MSR becomes exaggerated due to a lack of supraspinal inhibition, following a spinal cord injury for example, it can lead to tremor, clonus and hyperreflexia (Murray et al. 2010; Schmidt and Jordan. 2000). One source of inhibitory control over the MSR is 5-HT (Honda et al. 2004; Honda et al. 2006). We have shown previously that the activation of 5-HT_{1D} receptors with the agonist zolmitriptan, potently inhibits the MSR whereas no other 5-HT receptor influences sensory transmission in the MSR (Lucas-Osma et al. 2019). Interestingly, these 5-HT_{1D} receptors are expressed exclusively on C fibres (Lucas-Osma et al. 2019). Consequently, we investigate here how C fibres influence sensory transmission to motoneurons in the ventral horn and the mechanism behind this action.

C fibre activity has been shown to induce a long lasting depolarization of the large proprioceptive afferents involved in the MSR (tonic primary afferent depolarization, abbreviated tonic PAD, lasting many minutes or longer). This is mediated indirectly by the activation of GABAergic neurons that, in turn, activate extrasynaptic α5 $GABA_A$ receptors on the proprioceptive afferents (Lucas-Osma et al. 2018). Critically, this tonic PAD facilitates spike transmission in large diameter proprioceptive afferents (Lucas-Osma et al. 2018). This idea of a long lasting facilitatory tonic PAD is counterintuitive to the decades of classic research on presynaptic inhibition, where it has been posited that a more rapid phasic PAD (lasting ~ 100 ms) inhibits proprioceptive afferent transmission to motoneurons (Eccles et al. 1962; Rudomin and Schmidt. 1999; Wall. 1958). We know that all forms of PAD are mediated by GABAergic

interneurons that directly innervate afferent axons in the spinal cord and this modulates their transmission (classic PAD; Eccles et al. 1962; Rudomin and Schmidt. 1999; Russo et al. 2000). Specifically, it is thought GAD2 expressing GABAergic neurons are involved in PAD (Fink et al. 2014). This PAD is caused by the activation of $GABA_A$ receptors which causes an outward movement of chloride ions because of the unusually high intracellular chloride content of adult sensory afferents. This is due to these sensory afferents possessing the chloride transporter that brings chloride into cells (NKCC1; $Na^+/K^+/Cl^-$ cotransporter isoform 1; transports chloride into the cell), and mostly lacking the reverse transporter (KCC2; K^+/Cl^- cotransporter isoform 2; extrudes chloride from the cell; Chamma et al. 2012; Hasbargen et al. 2010). The outward chloride current causes a depolarization (PAD).

Axons themselves can activate the GABA neurons that then produce a fast phasic PAD in nearby afferents. For example, stimulation of low threshold proprioceptive afferents is well known to produce a fairly rapid PAD lasting less than 100 ms (Barron and Mathews. 1938; Eccles et al. 1962; Wall. 1958; Willis. 1999). This classic phasic PAD is thought to be mediated by a trisynaptic loop, where proprioceptive or cutaneous afferents synapse onto an excitatory glutamatergic neuron which spreads the excitation intersegmentally and commissurally. Then, in turn, this glutamatergic neuron synapses onto a GABAergic neuron, which then synapses back onto the proprioceptive afferent via axo-axonic contacts and this forms the classical trisynaptic loop of PAD (Delgado-Lezama et al. 2013; Eccles et al. 1962; Hughes et al. 2005; Rudomin. 2000).

Classically this phasic PAD has been closely associated with presynaptic inhibition of afferents when it was discovered that the extensor motoneuron MSR was strongly inhibited by a brief flexor nerve stimulation, with a time course similar to PAD evoked by the flexor nerve (Frank

and Fuortes. 1957; Willis. 1999). This presynaptic inhibition is mediated by GABAergic neurons, activating $GABA_A$ receptors and associated PAD, which is then thought to inhibit transmitter release to cause inhibition (Fink et al. 2014; Graham and Redman. 1994). However, phasic PAD can also have excitatory actions (Bos et al. 2011; Willis. 1999; Lucas-Osma et al. 2018), and in some cases phasic PAD evoked by a dorsal root stimulation can be so large that it directly excites proprioceptive afferents and produces spikes that travel antidromically out of the dorsal root, termed dorsal root reflexes (DRRs) (Eccles et al. 1961; Lucas-Osma et al. 2018; Rudomin and Schmidt. 1999). DRRs have been associated with several medical conditions including neurogenic inflammation and arthritis (Ferrell and Russell. 1986; Szolcsányi. 1988; Willis. 1999).

As mentioned previously, C fibres are particularly effective in eliciting a tonic PAD (Lucas-Osma et al. 2018). Interestingly, the α5 $GABA_A$ receptors which mediate this tonic PAD, are located at branch point nodes far from afferent terminals directly mirroring the location of Na^+ channels. It is at these branch points where, theoretically, propagating action potentials are most vulnerable to failure (Walmsley et al. 1995). We found previously that increasing proprioceptive afferent induced tonic PAD facilitates spikes causing less failure. In contrast, blocking tonic PAD has the opposite effect (Li et al. 2017; Lucas-Osma et al. 2018).

As detailed above, we have previously shown that the $5\text{-}HT_{1D}$ receptor agonist zolmitriptan potently inhibits the MSR and these 5-HT receptors are found exclusively on C fibres (Lucas-Osma et al. 2019). This indicates that somehow C fibres are able to influence sensory transmission to the ventral horn. Given our new findings demonstrating that proprioceptive afferent induced tonic PAD facilitates spikes and sensory transmission, and C fibres are particularly effecting at evoking this tonic PAD (Lucas-Osma et al. 2018), we propose that C

fibres are able to facilitate spike transmission by increasing tonic PAD and associated GABA. This ultimately leads to a reduced MSR when C fibres are inhibited via zolmitriptan. Here we wanted to investigate these ideas further and determine the possible circuitry of C fibres with GABAergic neurons. Parts of this work have already been published in abstract form (Li et al. 2016).

METHODS

Low threshold primary sensory afferents, including proprioceptive group Ia afferents were recorded to allow us to study phasic primary afferent depolarization (phasic PAD), tonic primary afferent depolarization (tonic PAD) and dorsal root reflexes (DRRs). Monosynaptic reflexes (MSRs) were evoked from dorsal root stimulation and recorded from ventral roots to allow us to see how the MSR changed in response to changes in C fibre activity. These experiments were conducted in the whole sacrocaudal spinal cord of adult female Sprague-Dawley rats (2 - 5 months old; > 200 g) maintained in vitro. All experiments were approved by the Health Sciences University of Alberta Animal Care and Use Committee.

In vitro preparation

Under urethane anesthesia (1.8g/kg with a maximum dose of 0.45g), a laminectomy was performed and the whole spinal cord from L2 to the Ca1 region was rapidly removed and placed in oxygenated modified artificial cerebrospinal fluid (mACSF) (see Lucas-Osma et al. 2018 for details). For ventral root reflex recordings, all spinal roots were removed apart from the sacral S4 and caudal Ca1 ventral roots for recording and the Ca1 or S4 dorsal roots for stimulation. For dorsal root recordings (all PAD experiments) all ventral roots were removed and S3, S4 and Ca1 dorsal roots were kept intact with usually two dorsal roots used for stimulation and four dorsal roots used for recording. For preparations involving intracellular recordings, the spinal cord was stabilised to a mesh piece of paper by gluing it ventral side down with a trace amount of cyanoacrylate. To record PAD and afferents in the deep dorsal horn, the spinal cord was glued so the left side of the cord was facing upwards to allow better access to the regions of interest. After

approximately 0.5 - 1 hour in mACSF the cord was transferred to a recording chamber containing normal ACSF (nACSF) saturated with carbogen (95% O_2 and 5% CO_2). This chamber was maintained near 21°C with a flow rate of approximately 5ml/min.

Intracellular recordings from afferents

To record from sensory afferents in the spinal cord, we used specialized ultra-sharp intracellular electrodes modified from those that we developed for recording motoneurons (Harvey et al. 2006; Lucas-Osma et al. 2018) to ensure that we didn't damage the fine afferent collaterals or disturb the intracellular milieu of the afferents we were recording from. Glass capillary tubes (1.5 mm outer and 0.86 mm inner diameters with filament; 603000 A-M Systems; Sequim; USA) were pulled using a Sutter P-87 puller (Flaming Brown; Sutter Instrument, Novato, USA) to make bee stinger electrodes. These electrodes have a moderately wide final shaft (approximately 1 mm) that tapered gradually from 30 to 3 μm in length, then abruptly tapered to a final tip over the last 20 μm length. The electrodes were filled through their tips with 1 M K-acetate mixed with 1 M KCl. Our previous work found that GABAergic chloride-mediated potentials (PAD) were the same despite different concentrations of KCl, indicating that the ultra-sharp electrodes we used impeded fluid exchange between the electrode and the cell, causing no disruption to the intracellular milieu (Lucas-Osma et al. 2018). Following this, the electrode tips were bevelled from an initial resistance of 40 – 150 MΩ to 30 – 40 MΩ using a rotary beveller (Sutter BV-10). Electrodes were advanced into afferents using a stepper motor (666, Kopf, USA, 10 μm steps at maximal speed), typically at the boundary between the dorsal columns and the grey matter, but occasionally also deeper in the dorsal horn. All intracellular recordings were made using an

Axoclamp2B amplifier (Axon Instrument and Molecular Devices, San Jose, USA) and sampled at 30 KHz (Clampex and Clampfit; Molecular Devices, San Jose, USA).

Dorsal root stimulation, afferent identification and recording

Dorsal roots were mounted on silver-silver chloride wires above the recording chamber and covered with a 3:1 mixture of petroleum jelly and mineral oil respectively, surrounded by a high vacuum synthetic grease barrier. The dorsal roots were stimulated with a current pulse (0.1 ms) with varying intensities, expressed as a multiple of the afferent volley threshold (T) of approximately 0.003 mA. When the intracellular recording electrode was in the dorsal horn, an extracellular field corresponding to the group Ia afferent volley was observed as the first event following dorsal root stimulation. This occurred with a latency of 0.5 – 1.0 ms, depending on the root length (the roots were kept as long as possible, 10 – 20 mm), corresponding to a conduction velocity of approximately 16 – 24 m/s, as previously described for room temperature in vitro conduction (Li et al. 2004). Our focus centred on the lowest threshold afferents, mainly proprioceptive group Ia afferents. These were identified by their direct response (spike) to dorsal root stimulation, short latency (group Ia coincident with onset of afferent volley), very low threshold (<1.5 x T) and antidromic response to ventral horn afferent terminal microstimulation (approximately 10 μA stimulation via tungsten microelectrode to activate Ia afferent terminals; tested in some afferents; discussed in more detail later in the methods). Good quality afferents used for analysis rested near – 70 mV, had a spike that peaked at about + 10 mV (overshoot) and had a brief afterhyperpolarization (10 ms). We were then able to record their electrophysiological properties, including PAD and antidromic spikes evoked by this PAD. In addition, once we penetrated and identified a Ia afferent we were able to record its membrane potential over a long

time course. Once a steady baseline was achieved we electrically stimulated C fibres (11 stims, 1 Hz) at 50 x T and recorded the change, if any, in the Ia afferent membrane potential (see details below on selectively stimulating C fibres). Moreover, we stimulated C fibres chemically via the application of specific drugs to the recording chamber to activate TRP receptors found on C fibres. Icilin is a transient receptor potential melastatin 8 (TRPM8) receptor agonist; these receptors detect menthol and cold stimuli (Todd. 2002) and are activated at approximately 22°C and inactivated at approximately 26°C - 27°C (Andersson et al. 2004). Zolmitriptan, as mentioned in the introduction, is a 5-HT_{1D} receptor agonist, with these receptors found exclusively on C fibres (Lucas-Osma et al. 2018; Potrebic et al. 2003). We used a 3 or 10 nM dose of zolmitriptan as a low dose only activates 5-HT_{1D} receptors as opposed to higher doses which also activate 5-HT_{1B} receptors. We added icilin (10 μM) or zolmitriptan (3 or 10 nM) to the recording chamber and recorded the effects on the intracellular Ia afferent membrane potential. From our initial results we found that zolmitriptan is a useful drug to inactivate C fibres (see results section) and therefore we were able to use this drug to determine what effect C fibre inactivation had on several different modalities.

We also recorded from the central ends of dorsal roots cut within a few mm of their entry into the spinal cord to give the compound potential from all afferents in the root (dorsal root potential; DRP). The DRP has previously been shown to correspond to PAD, though it is attenuated compared to intracellular recordings of PAD. We found that when we wanted to record the slower components of PAD (tonic PAD) lasting many seconds or minutes, the silver-silver chloride electrodes (detailed above), sometimes had too much electrical drift at the electrode-liquid interface to be reliable. Thus we developed a miniaturized grease gap recording method to solve this problem. For this we filled one end of a 1.5 mm glass capillary tube

(without filament; 628500; A-M Systems, Sequim, USA) with ACSF (or 100 μM KCl) mixed with 1.5 % agar and mounted the dorsal root onto the agar end of the tube and sealed the root in grease just above the bath (again using short roots close to the dorsal root entry zone into the spinal cord). The other end of the capillary tube was filled with 1 M NaCl and a silver-silver chloride wire was inserted into this fluid to create a highly conductive liquid-metal interface, reducing the slow drift in the electrode-liquid interface. We then recorded this signal with a DC coupled amplifier (Axoclamp2B amplifier, Axon Instruments, Molecular Devices, San Jose, USA; low pass filtered at 3 kHz) to allow us to record the DRP (tonic PAD) without extensive drift.

The rapid phasic PAD and associated evoked spikes (DRRs) were usually recorded from dorsal roots attached to the sacrocaudal spinal cord mounted on silver-silver chloride wires above the nACSF of the recording chamber and covered with a 3:1 mixture of petroleum jelly and mineral oil (Li et al. 2004). As detailed above, it was imperative that we used very short dorsal roots cut very close to their dorsal root entry zone of the cord (within 500 μm) for this recording, so that the passive attenuation of PAD was minimized. For these experiments all ventral roots were removed and S3-Ca1 dorsal roots were kept intact with usually two dorsal roots used for stimulation and four dorsal roots used for recording purposes (standard dorsal root recording setup). This allowed us to record antidromic spikes evoked by PAD (DRRs) and phasic PAD evoked by the stimulation of one dorsal root and recorded on an adjacent dorsal root. For phasic PAD we used a custom amplifier to low pass filter the data at 3 kHz and high pass filter the data at 0.1 Hz, digitized at 30 kHz. Following dorsal root stimulation at Ia intensity (2 x T), phasic PAD arised approximately 10 ms after this stimulation with DRRs occurring on the rising phase of phasic PAD. This phasic PAD lasted approximately 100 ms. We added the drugs icilin (10

μM) or zolmitriptan (3 nM) to the spinal cord in the recording chamber to determine how C fibre activation or inactivation affected this phasic PAD and DRRs. Using the grease gap method detailed above, we also recorded the afferent potentials (DRPs) over long periods (tonic PAD) to see how it changed with the application of icilin (10 μM), capsaicin (10 μM) and zolmitriptan (10 nM) using Axoclamp 2B amplifier (Axon Instruments, Molecular Devices, San Jose, USA; low pass filtered at 3 kHz, DC coupled without high pass filter). Capsaicin activates the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor, which detects noxious heat stimuli on C fibres (Levine and Alessandri-Haber. 2007; Spicarova et al. 2014).

Ventral horn microstimulation

A method to directly assess proprioceptive afferent conduction is to selectively stimulate their terminals in the ventral horn and record the direct propagation of spikes into the dorsal roots, similar to the approaches developed by Rudomin and Wall (Rudomin and Schmidt. 1999; Wall. 1958). We did this with a tungsten microelectrode placed in the S4 ventral horn that we stimulated minimally to reduce current spread (at ~ 7 μA, 2 x T, 10 MΩ) and we recorded the compound action potentials that managed to propagate antidromically to the dorsal root with our standard dorsal root recording arrangement, detailed previously. From the evoked compound action potential we assessed the earliest phase to avoid later slow afferents activated by current spread. In this way, the amplitude of the compound action potential reflects the number of axons stimulated that did not fail to propagate spikes to the dorsal root. We then added zolmitriptan (3 nM) to assess its effects on the compound action potential as well as adding bicuculline (50 μM; competitive antagonist of $GABA_A$ receptors) to assess the role of GABA.

Extracellular field recordings

Extracellular fields from the spinal cord were recorded by penetrating the spinal cord at the site of interest with sharp microelectrodes. Dorsal roots were hooked up to silver-silver chloride wires for stimulation in the main chamber to allow us to stimulate large proprioceptive sensory afferents, and we recorded the evoked extracellular field using the microelectrodes. We penetrated the cord at different locations; namely the dorsal horn and the ventral horn, and we recorded the extracellular fields evoked from the fast compound action potentials evoked in large proprioceptive sensory afferents by dorsal root stimulation (at 1 – 1.5 x T, fastest component of field). To establish the expected timing of these fields, we also directly penetrated large proprioceptive afferents in the dorsal columns to directly measure the action potential (AP; spike) evoked by dorsal root stimulation. The proprioceptive afferent field potentials had a triphasic shape: I, an initial positive phase caused by passive current driven by distal APs; II, a prominent negative phase (volley) caused by the leading edge of the AP as it reaches the recording site; III, a late positive phase (tail current) caused again by passive current but from the AP propagating past the recording electrode. It is the negative extracellular field (phase II) that allowed us to see how sensory transmission changed with C fibre activation, as it is this component that relates to the size of the intracellular AP. As such, these extracellular field recordings can give us an indication of how well the APs are propagating down to the motoneurons in the ventral horn.

Ventral root reflex recordings

The monosynaptic reflex (MSR) was recorded from ventral roots of the sacrocaudal spinal cord. Dorsal and ventral roots were mounted on silver-silver chloride wires above the nACSF of the recording chamber and covered with a 3:1 mixture of petroleum jelly and mineral oil for monopolar stimulation and recording, as we detailed previously (Li et al. 2004), though with shorter ventral roots to help record the underlying EPSP, as well as the reflexes (spikes). Ventral roots on both sides of the spinal cord were mounted for recording purposes, this included S3, S4 and Ca1 caudal ventral roots. We evoked ventral root reflexes in these sacral roots with a low threshold stimulation of the ipsilateral Ca1 or ipsilateral S4 dorsal root at T, where afferent and reflex threshold are similar (Bennett et al. 2004; single pulses, 0.1 ms, ~0.02 mA) using a constant current stimulator (Isoflex, Israel). The stimulation was repeated 5 times at 10 s intervals for each trail then this recording procedure was repeated every 15 minutes. The recordings were amplified (x 2000), high pass filtered at 0.1 or 100 Hz, low pass filtered at 3 kHz, and recorded with a data acquisition system sampling at 6.7 kHz (Axoscope 8, Axon Instruments). Following dorsal root stimulation an afferent volley arrived at the spinal cord approximately 0.8 – 1 ms after (due to root conduction delay), and then the MSR arose approximately 1 ms later (the synaptic delay is about 1 ms in vitro at room temperature), and therefore the MSR latency is approximately 2 ms following dorsal root stimulation. Once a steady baseline was achieved, we added icilin (10 μM) to activate C fibres and recorded the MSR. Moreover, we stimulated C fibres electrically by stimulating the dorsal roots at 50 x T (5 ms pulse width, detailed below) and then 10 - 20 s later we recorded the MSR evoked by a low threshold dorsal root stimulation to determine how the preceding C fibre stimulation effected the MSR. We were confident that we were indeed recruiting C fibres with this high threshold

stimulation because the overall response increased when compared to stimulation at Ia afferent intensity (2 - 3 x T). Furthermore, we also isolated the C fibre stimulation as detailed next.

Selective electrical stimulation of C fibres

We developed three methods to selectively activate C fibres, each of which had its limitations, but we usually combined these methods to achieve just C fibre activation. First, we maintained a very long length (~30 mm) of a dorsal root in a bath adjacent to the main bath with the spinal cord, separated by a grease barrier and perfused with a low dose of tetrodotoxin (TTX, referred to as low TTX side bath) to the spinal cord (100 - 200 nM). This has been shown to eliminate spike generation in all sensory axons except unmyelinated C fibres that have TTX-resistant sodium channels (Russo et al. 2000; Sangameswaran et al. 1996; Tate et al. 1998; Yoshida et al. 1978). We then stimulated the distal end of this dorsal root at 50 x T to activate C fibres. In control experiments where we recorded the central end of this long dorsal root (on silver wire in grease as detailed above), we found that as the low dose TTX was applied to the side-bath all but the slow C fibre mediated volleys were eliminated, confirming selective C fibre activation, though some portion of the C fibre volley was also usually eliminated as the dose approached 200 nM (not shown); thus a minimal dose was used that may have had small residual fast non C fibre activation. Second, we stimulated the dorsal root in the side bath with a bipolar electrode made from gluing two fine tungsten stimulating electrodes (10 MΩ) together so that their tips were separated by only 50 μm. This arrangement readily activated the unmyelinated C fibres, but considerably raised the threshold for activation of large myelinated afferents where the internodal spacing is near 1 mm (Scurfield et al. 2018), thus minimizing non C fibre activation in

the TTX side bath. The long length of the dorsal root in the side bath minimized passive current spread down the large axons into the main bath lacking TTX. Third, we developed a cathodal block circuit to stimulate C fibres while blocking larger axons, where the applied current was raised slowly over 5 ms (with a capacitive coupling) to impose a cathodal block to nearby nodes in large afferents, and then the dorsal root was stimulated on the descending negative phase of this current, which tended to favour just slow C fibre activation, as again confirmed in control experiments recorded from the central end of the root in grease (not shown).

In some experiments where we wanted to record from C fibres in the dorsal root in isolation we completely blocked all spikes including C fibre spikes with a high dose of TTX (2 μM) and eliminated fast synaptic transmission with CNQX (10 μM), a AMPA/kainite receptor antagonist; AP5 (50 μM), a NMDA receptor antagonist; strychnine (5 μM), a glycine receptor antagonist; and gabazine (10 μM), a $GABA_A$ receptor antagonist. In this case the C fibres were activated chemically as detailed above.

Drugs and solutions

Two types of artificial modified cerebrospinal fluid (ACSF) are used during in vitro experiments. Modified artificial cerebrospinal fluid (mACSF) was used in the dissection dish and the normal artificial cerebrospinal fluid (nACSF) was used in the recording chamber. The mACSF was composed of (in mM) 118 NaCl, 24 $NaHCO_3$, 1.5 $CaCl_2$, 3 KCl, 5 $MgCl_2$, 1.4 NaH_2PO_4, 1.3 $MgSO_4$, 25 D-glucose, and 1 kynurenic acid. The nACSF was composed of (in mM) 122 NaCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 3 KCl, 1 $MgCl_2$, and 12 D-glucose. Both types of ACSF were saturated with carbogen (95% O_2 and 5% CO_2) and maintained at a pH of 7.4. The drugs added to the

nACSF were capsaicin, icilin, gabazine, CNQX, AP5, bicuculline (all from Tocris, Minneapolis, USA) and TTX (TRC, Toronto, Canada). All drugs were dissolved as a 10 – 50 mM stock in distilled water before final dilution in nACSF and applied to the spinal cord in vitro. The drugs zolmitriptan and strychnine (both from Tocris, Minneapolis, USA) were first dissolved in a minimal amount of DMSO (final concentration in nACSF of 0.02 %; by itself DMSO has no effect on in vitro DRP, DRRs or MSRs in vehicle controls) in order for the drug to fully dissolve, which was then added to the nACSF and added to the spinal cord in the recording chamber.

Genetically labelling GAD2 neurons

We have bred a strain of mice with GAD2 neurons, a specific subpopulation of GABAergic neurons, labelled with a fluorescent reporter; enhanced green fluorescent protein (EGFP), to visualize GAD2 neurons. Breeding pairs of Tamamaki CL-1 white albino Swiss mice were obtained from Dr. Jean Claude LaCaille (University of Montreal, Montreal QC, Canada) and bred in the Health Sciences Laboratory Animal Services facility at the Univeristy of Alberta (Tamamaki et al. 2003). These mice labelled GAD-67 and were crossed with reporter mice with floxed stop codons on sequences for EGFP (flx-EGFP mice) to create GAD-cre-ER mice. The mice are given tamoxifen to induce cre (2 x 4 mg intraperitoneal) at 4 weeks old.

Immunolabelling

Transgenic mice, outlined above, were euthanized with Euthanyl (BimedaMTC; 700 mg/kg) and perfused intracardially with 100 ml of saline containing sodium nitrite (1 g/l; Fisher) and heparin (300 IU/l, from 1,000 U/ml stock; Leo Pharma) for 3 - 4 minutes, followed by 400 ml of 4%

paraformaldehyde (PFA; in phosphate buffer at room temperature), over 15 minutes. Spinal cords were postfixed in PFA overnight at 4°C and then immersed in 30 % sucrose in phosphate buffer for 72 hours. The spinal cord tissue was coated with O.C.T cryoprotectant (Sakura Finetek) before being frozen in 2-methylbutane (Fisher Scientific) and then cut on a cryostat NX70 (Fisher Scientific) in transverse 25 µm sections. The tissue was stored at -20°C until histological processing. We mounted the spinal cord sections on slides and rinsed with Tris-buffered saline (TBS, 50 mM) containing 0.3% Triton X-100 (TBS-TX, 10 minute rinses used for all TBS-TX rinses). Next, nonspecific binding was blocked with a 1 hour incubation in 10 % normal donkey serum (NDS; ab7475, Abcam) in PBS-TX. The primary antibodies: sheep anti-calcitonin gene-related peptide (CGRP; 1:5000; ab22560, Abcam), mouse anti-Bassoon (1:400; SAP7F407, ENZO) and rabbit anti GFP (1:500; A11122, Life Technology), were diluted in 2 % NDS in PBS-TX and applied to slides overnight at room temperature. The following day, the tissue was rinsed with PBS-TX (3 x 10 mins) and incubated with fluorescent secondary antibodies: donkey anti-sheep Alexa Fluor 647 (1:500; ab150179, Abcam), donkey anti-mouse Alexa Fluor 555 (1:500; ab150106, Abcam) and donkey anti-rabbit Alexa Fluor 488 (1:500; A-21206, Thermofisher) diluted in 2% NDS in PBS-TX for 2 hours at room temperature. After rinsing with PBS-TX (2 x 10 mins) and PBS (2 x 10 mins), the slides were covered with Fluoromount-G (00-4958-02, ThermoFisher Scientific, Waltham, USA) and coverslipped in Permount (Sakura Finetek USA, Torrance, CA, USA).

Standard negative controls in which the primary antibody with either 1) omitted or 2) blocked with its antigen (quenching) were used to confirm the selectively of the antibody staining, and no specific staining was observed in these controls. For antibody quenching, the peptides used to generate the antibodies (AAP34984, Aviva Systems Biology, San Diego, USA) were mixed with

the antibodies at a 10:1 ratio and incubated for 20 hours at 4°C. This mixture was then used instead of the antibody in the above staining procedure.

Image acquisition

Image acquisition was performed by epifluorescence (Leica DM 6000 B) for low magnification imaging and confocal microscopy (Leica TCS SP8 Confocal System) was used for high magnification 3D reconstruction (Fig 2.9; for more details see Lucas-Osma et al. 2018). Leica Application Suite X (Leica Microsystems CMS GmbH) was used for visualization and analysis of all images taken.

Data analysis

To effectively quantify PAD we measured the compound effects of PAD by measuring the dorsal root potential (DRP) from the cut end of dorsal roots to allow us to record steady DC potentials without extensive drift (Figs 2.1B-C; 2.4B- F; 2.5A). This phasic DRP has an identical time course to phasic PAD but was an order of magnitude smaller in absolute potential than the average phasic PAD recorded intracellularly (Lucas-Osma et al. 2018). We were able to efficiently determine the effects of drugs on the afferent membrane potential by inferring tonic PAD relative to the changes in the DRP. We did this by taking the membrane potential before the drug of interest was added, divided by the size of phasic PAD for that root and then multiplying by the average size of intracellular PAD which is approximately 3.7 mV (Lucas-Osma et al. 2018). We then completed the same calculation but using the membrane potential after the drug of interest was added and then we were able to calculate the change in membrane potential

caused by a specific drug from these values by subtracting one from the other (Figs 2.1D; 2.4D and G; 2.5B).

Data was analyzed using Clampfit 8.0 (Axon Instruments, USA) and Sigmaplot (Jandel Scientific, USA) and is shown as a mean ± standard deviation (SD, the latter used to quantify variability). A paired *t*-test was used to test for statistical differences, with a significance level of $P < 0.05$.

RESULTS

C fibres are activated by icilin and inhibited by zolmitriptan

To study C fibres in isolation we bathed the whole spinal cord in TTX (2 μM) and synaptic blockers (10 μM CNQX, 50 μM AP5, 5 μM strychnine, 10 μM gabazine), while recording the central cut ends of dorsal roots in the whole isolated in vitro adult rat spinal cord. When we then activated C fibres chemically using the TRPM8 agonist icilin (10 μM; Andersson et al. 2004; Mergler et al. 2007), this depolarized the dorsal roots (dorsal root potential, DRP; Fig 2.1B) indicating an overall depolarization of C fibres since TRP receptors are exclusively found on C fibres (Richter et al. 2019; De- Schepper et al. 2008). In contrast, the selective 5-HT_{1D} receptor agonist zolmitriptan (3 nM; Tepper et al. 2002; Thomsen et al. 1996) hyperpolarized the DRP (Fig 2.1C). Because these 5-HT receptors are found exclusively on C fibres (Lucas-Osma et al. 2019; Potrebic et al. 2003), this indicates that the C fibres were hyperpolarized by zolmitriptan. Overall, these results demonstrate that zolmitriptan and TRP agonists like icilin are effective tools for inactivating or activating C fibre actions, respectively, which we used extensively throughout our experiments.

C fibre activity increases the MSR to motoneurons

We next examined whether C fibres modulated sensory transmission to motoneurons by recording the monosynaptic reflexes (MSRs) from ventral roots in response to dorsal root stimulation at proprioceptive sensory afferent intensity (2 x T). When we selectively stimulated C fibres electrically (stimulating the dorsal root at 50 x T while the root was bathed in low dose TTX to block all but C fibre conduction; detailed in Methods), the MSR recorded 10 – 20 s

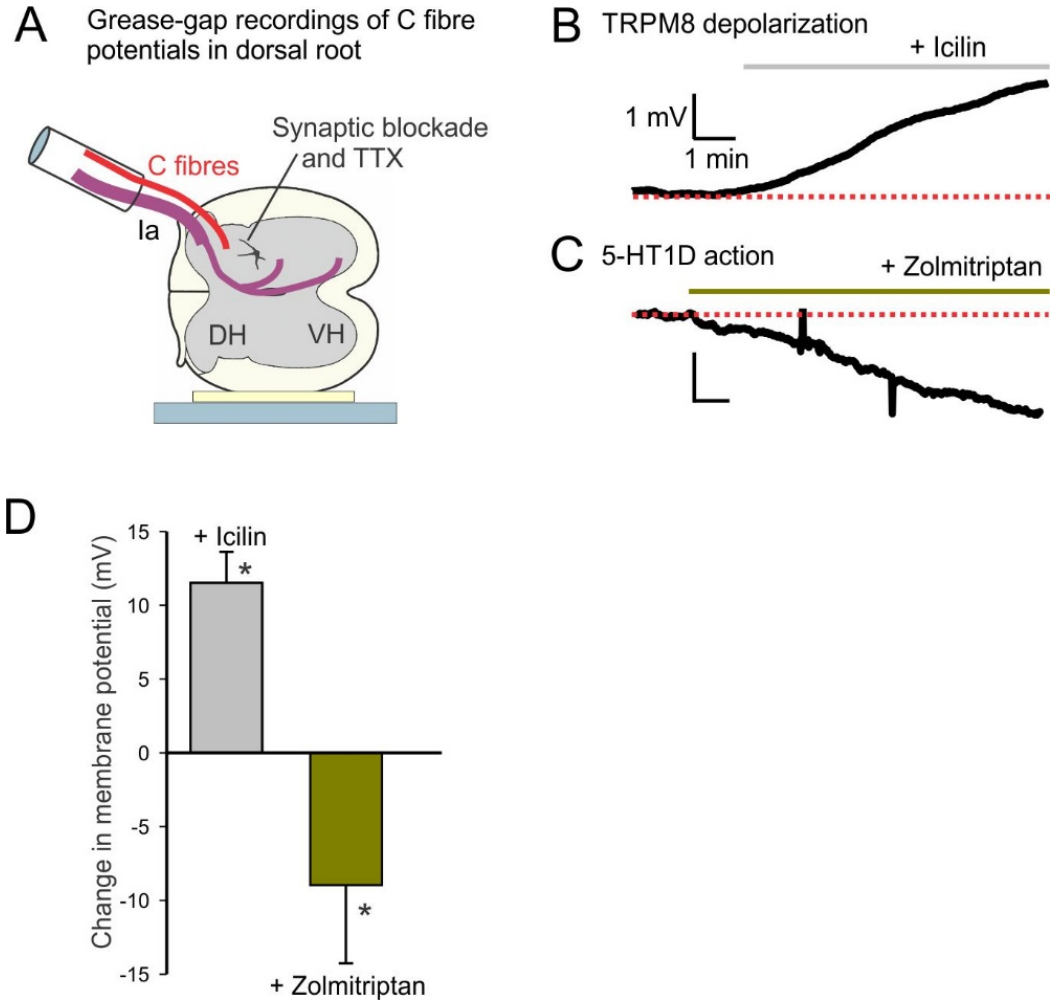


Figure 2.1. Icilin depolarizes C fibres whereas zolmitriptan hyperpolarizes C fibres, demonstrating that these drugs are useful tools to either activate or inactivate C fibres.

A: Recording of C fibre potential changes in dorsal roots in the presence of TTX (2 µM) and full synaptic block (10 µM CNQX, 50 µM APV, 5 µM strychnine and 10 µM gabazine) to block all spike transmission. **B:** Application of the TRPM8 receptor agonist icilin (10 µM) depolarized C fibres in the presence of full synaptic block. **C**: Activation of 5-HT_{1D} receptors via zolmitriptan (3 nM) hyperpolarized C fibres in the presence of full synaptic block. **D**: Following the application of icilin to the spinal cord the C fibres depolarized (n = 5), whereas following the application of zolmitriptan (n = 5) the C fibres hyperpolarized in the presence of TTX and full synaptic block, shown as the change in compound membrane potential relative to control. Error bars SD. * significantly different, $P < 0.05$.

later was strongly facilitated (Fig 2.2B), without altering motoneuron activity at these long delays (i.e. pre and not postsynaptic facilitation). This MSR facilitation built up with repeated C fibre stimulation (at 10 s intervals) and lasted several minutes after stimulation ceased (Fig 2.2C). Likewise, chemical activation of C fibres with icilin (10 μM) facilitated the MSR (Fig 2.2D), again without altering motor output prior to MSR testing. In contrast, the application of zolmitriptan markedly reduced the MSR and associated EPSP (Fig 2.2E), and blocked the facilitation of the MSR by icilin (Fig 2.2D). As we have previously reported, zolmitriptan has no direct action on motoneurons (Murray et al. 2011), suggesting again that it acts presynaptically. Overall, this suggests that C fibres somehow affect proprioceptive afferent transmission to motoneurons, and even do so spontaneously (prior to the application of zolmitriptan).

Many of our experiments used icilin to activate C fibres. This drug activates TRPM8 receptors which, as outlined in the methods, respond to cool temperatures, turning on near room temperature (around 22°C) where we normally maintain our in vitro bath. Interestingly, when we warmed the nACSF in the bath to 28°C to turn off the TRPM8 receptors, we found that the MSR was strongly inhibited (Fig 2.2F), consistent with there being a tonic C fibre TRPM8 receptor facilitation of the MSR, as shown by an increase in the MSR with icilin, though other temperature effects (Brooks et al. 1955; Toennies 1939) may confound this simple experiment.

C fibre activity facilitates sensory transmission to the ventral horn

To confirm that C fibre activation increases proprioceptive afferent transmission in the spinal cord, we directly measured from sensory afferents to quantify how C fibre activation affects sensory transmission to the ventral horn. Using sharp microelectrodes we penetrated the cord at

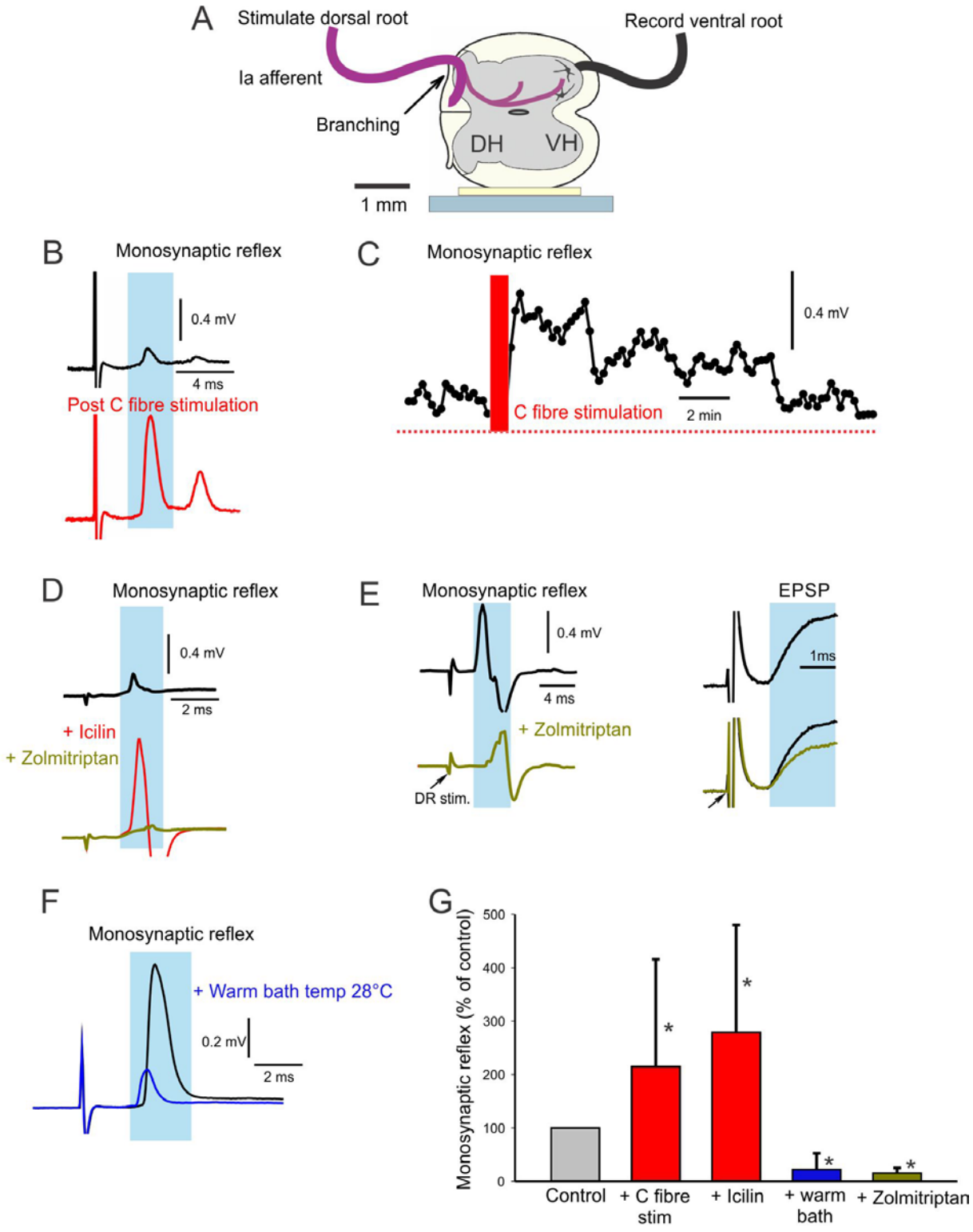

A
Stimulate dorsal root
Record ventral root
Ia afferent
Branching
DH
VH
1 mm
B
Monosynaptic reflex
0.4 mV
4 ms
Post C fibre stimulation
C
Monosynaptic reflex
0.4 mV
C fibre stimulation
2 min
D
Monosynaptic reflex
0.4 mV
2 ms
+ Icilin
+ Zolmitriptan
E
Monosynaptic reflex
0.4 mV
4 ms
+ Zolmitriptan
DR stim.
EPSP
1ms
F
Monosynaptic reflex
+ Warm bath temp 28°C
0.2 mV
2 ms
G
Monosynaptic reflex (% of control)
500
400
300
200
100
0
Control
+ C fibre stim
+ Icilin
+ warm bath
+ Zolmitriptan

Figure 2.2 Monosynaptic reflexes (MSRs) are facilitated by C fibre activation and inhibited by C fibre inactivation.

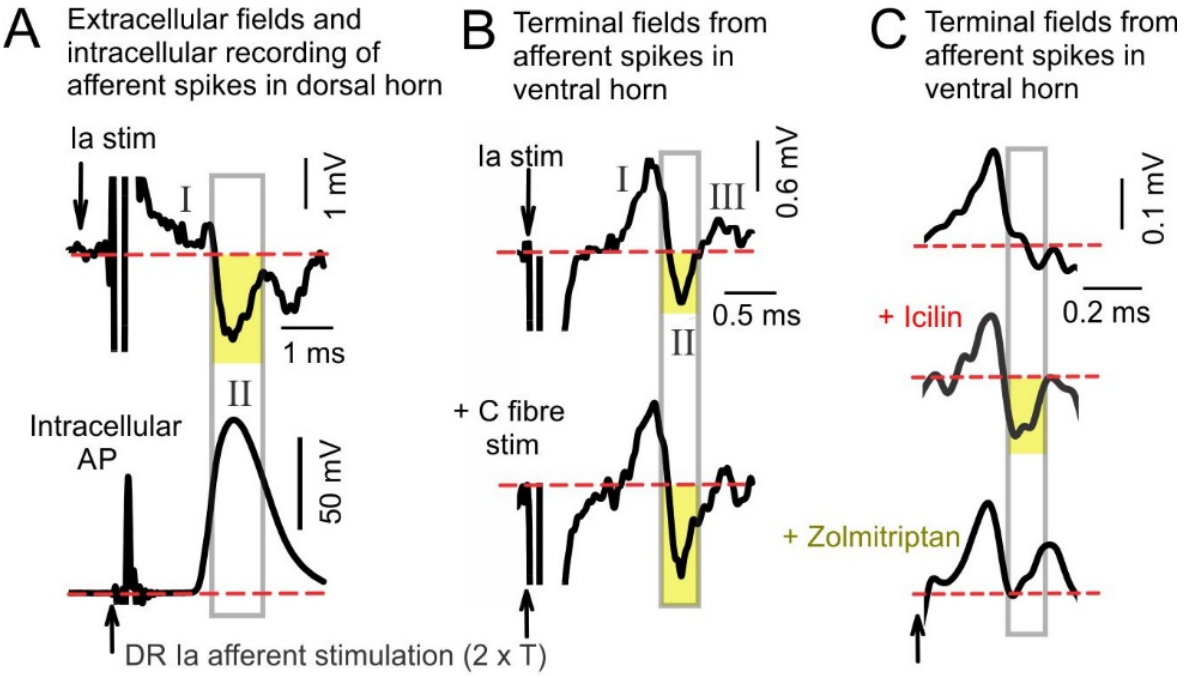
A: MSR is recorded from a ventral root evoked from a dorsal root stimulation (2 x T, 0.1 ms pulse). **B**: C fibres are isolated in a side bath containing low dose TTX (100 – 200 nM). The MSR was measured in the main recording chamber prior to C fibre stimulation and then measured again 20 s following C fibre stimulation (50 x T, at 20 s intervals). MSR is strongly facilitated following C fibre stimulation. Ventral root activity was not significantly different from zero with and without C fibre stimulation (not shown; $p > 0.05$, n = 19). **C**: Brief repeated C fibre stimulation (11 stims, 0.3 Hz, 50 x T) facilitated the MSR for several minutes (n = 19). **D**: The application of icilin (10 μM) to chemically activate C fibres, facilitated the MSR but did not change motoneuron activity (not shown; $p > 0.05$, n = 19), whereas the application of zolmitriptan (3 nM) after icilin reduced the MSR (n = 2). **E**: Zolmitriptan (3 nM) reduced the MSR and associated monosynaptic EPSP. **F**: MSR decreased when the bath temperature was raised to 28°C to inactivate TRPM8 receptors. **G**: On average, C fibre stimulation (n = 15) and icilin (n = 10) increased the MSR, whereas the application of zolmitriptan (n = 5) and warming the bath to 28°C, reduced the MSR (n = 5), seen as a percentage of MSR control. Background activity was absent prior to MSR testing (left of B - F), and not altered with these drugs. Error bars SD. * significantly different, $P < 0.05$.

different locations; namely the dorsal horn and the ventral horn, and we recorded extracellular fields evoked from the fast compound action potentials evoked in large proprioceptive sensory afferents by dorsal root stimulation (at 1 – 1.5 x T, fastest component of field). To establish the expected timing of these fields we also directly penetrated large proprioceptive afferents in the dorsal columns to intracellularly record the action potential (spike) evoked by dorsal root stimulation. The extracellular proprioceptive afferent field potentials had a triphasic shape: I, an initial positive phase caused by passive current driven by distal action potentials; II, a prominent negative phase (volley) caused by the leading edge of the action potential as it reaches the recording site; III, a late positive phase (tail current) caused again by passive current, but from the action potential propagating past the recording electrode (Fig 2.3A-C; as in Hubbard et al. 1971). It is the negative extracellular field (phase II) that allowed us to estimate how sensory

transmission changed with C fibre activation, as it reflects the size of the compound action potentials reaching the electrode from multiple nearby afferents and was the same latency as the intracellularly recorded action potential. When we measured this afferent field at the afferent terminals in the ventral horn of the spinal cord (and so called terminal afferent field), selective C fibre stimulation (50 x T as detailed above; at 1 Hz for 11 s) increased the Ia afferent terminal potential negative volley (phase II) for several minutes (Fig 2.3B) suggesting that C fibre activation increased action potential propagation to proprioceptive afferent terminals on the motoneurons (likely by preventing conduction failure, see Introduction). Similarly, activating C fibres via the application of icilin (10 μM) increased the Ia afferent terminal potential negative volley (phase II), whereas inactivating C fibres via the application of zolmitriptan (10 nM) reversed icilin's facilitatory effect and decreased the terminal potential negative volley (Fig 2.3C), and zolmitriptan itself decreased the Ia afferent terminal potential negative volley (Fig 2.3D). Overall, this suggests that C fibres somehow facilitate spike conduction and blocking them with zolmitriptan induces spike conduction failure.

To further quantify the effects of C fibres on proprioceptive afferent conduction failure to the ventral horn, we normalized the amplitude of the negative phase of the field (phase II, denoted n) by the total peak-to-peak size of the field (n + p, where p is the positive phase I amplitude) to compute a Conduction Index (CI) = n / (n + p). Prior studies have shown that failing spikes lose the negative phase II volley, but retain some of the positive phase I, from the passive effect of spikes at locations prior to failure (Hubbard et al. 1971), giving some indication of the total spikes prior to the failure point. This CI provided us with an index approximating the proportion of non-failing spikes: where CI 1 = no failure and 0 = complete failure. Being a ratio, this index also compensates for the large variability in the absolute afferent volley size between animals.

We found that following activation of C fibres (with icilin or by electrical stimulation), the conduction index increased, whereas inhibition of C fibres (with zolmitriptan) consistently decreased the conduction index (Fig 2.3D). Overall, these results show that C fibre activation increases sensory axon conductance to the ventral horn, consistent with the increased MSR transmission we observe with C fibre activation (Fig 2.2).



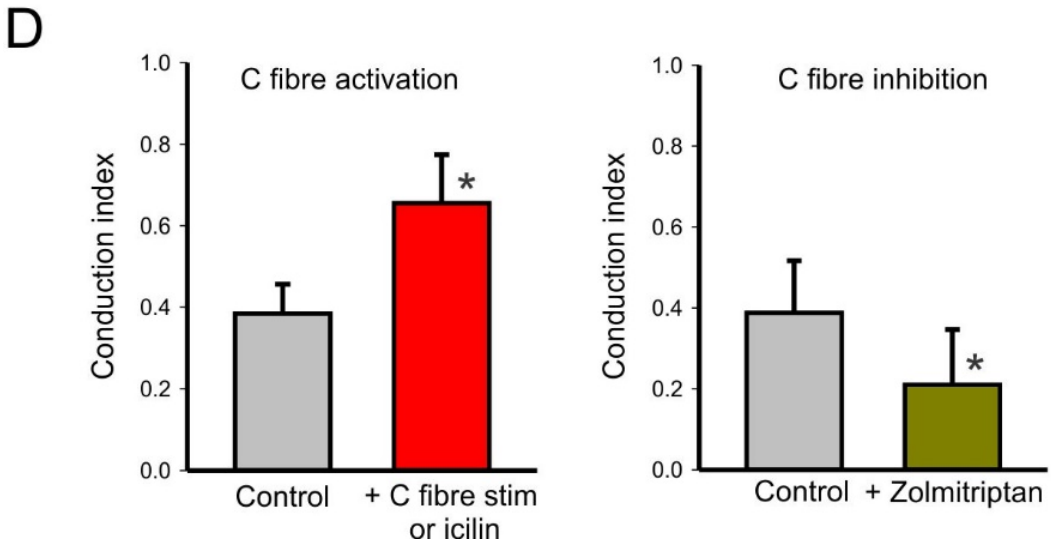


Figure 2.3. Activation of C fibres increases sensory transmission to the ventral horn.

A: Extracellularly recorded field potential (top) from compound action potentials evoked in large proprioceptive afferents by dorsal root stimulation (at 2 x T, 0.1 ms pulse), and compared to action potential (spike) recorded intracellularly by penetrating a nearby afferent after recording the extracellular field (bottom; n = 20). Recordings from dorsal horn near dorsal columns. **B**: Extracellular fields evoked again by stimulation of large proprioceptive afferents in the dorsal root (2 x T), but in this case recorded in the ventral horn at the afferent terminals (thus called terminal extracellular fields). The fields (in A and B) usually had a triphasic shape: I, an initial positive phase caused by passive current driven by distal action potentials (spikes); II, a prominent negative phase (volley, yellow) caused by the leading edge of the spikes as they reach the recording site; and III, a late positive phase (tail current) caused again by passive current but from the spikes propagating past the recording electrode (this latter phase is sometimes obscured by synaptic events). Selective C fibre stimulation (50 x T, 5 ms pulses at 1 Hz, 11 pulses, detailed in methods) of a nearby dorsal root (S4 typically), increased the proprioceptive afferent negative volley (phase II) for several minutes (averages of > 60 recordings over 1 – 2 minutes post C fibre stimulation, consistent with increased afferent conduction). **C**: Terminal extracellular fields again recorded as in B. Icilin (10 μM) increased proprioceptive afferent negative volley (phase II), which is consistent with C fibre activation. Zolmitriptan (10 nM) reduced icilin's facilitatory effect, eliminating the negative volley (phase II). **D**: To quantify these actions of C fibres on the terminal field in the ventral horn, we used the positive (*p, phase I*) and negative (*n, phase II*) fields of the triphasic shape of the afferent terminal extracellular field to compute a *conduction index = n / (n+p)*. Following the activation of C fibres (by stimulation at 50 x T, n = 2 or icilin, n = 3; combined), the conduction index increased, whereas inhibition of C fibres with zolmitriptan decreased the conduction index (n = 6). Error bars SD. * significantly different, $P < 0.05$.

C fibre activation increases proprioceptive afferent transmission via an α5 $GABA_A$ receptor induced tonic PAD

We next examined how C fibres increase afferent transmission, by recording intracellularly from afferents and specifically focusing on their activation of GABAergic systems. We have shown previously that C fibre activation elicits a long lasting GABA-mediated depolarization of large proprioceptive afferents (tonic PAD, produced by α5 $GABA_A$ receptors; Lucas-Osma et al.

2018), and this facilitates sensory axon transmission by preventing axon conduction failure (Lucas-Osma et al. 2018). Thus, we further examined here how C fibres modulate tonic PAD. When recording intracellularly from large proprioceptive afferents we found that indeed C fibre stimulation (50 x T, in isolation as above, or in partial isolation with slow bipolar pulses as detailed in Methods; 11 pulses at 1 Hz) directly depolarized the afferents (tonic PAD; Fig 2.4A), and inactivating C fibres with zolmitriptan hyperpolarized the afferents (Fig 2.4F). The latter demonstrates that not only do C fibres cause tonic PAD, but they must also be spontaneously active, steadily facilitating tonic PAD and associated sensory transmission.

To further quantify the modulation of PAD by C fibres, we recorded the composite PAD from many afferents on the central cut end of dorsal roots (DRP). Again we found that selective C fibre stimulation (in TTX side bath) or chemical activation with icilin or capsaicin, depolarized the afferents (Fig 2.4B, E), consistent with depolarization of large proprioceptive afferents. This dorsal root recording approach to study overall afferent depolarization is possibly contaminated by direct contribution from C fibres in the recording, though the bulk of the recorded signal is likely due to depolarization of large diameter afferents (like proprioceptors), simply because of their relative size compared to C fibres, and because the depolarization often rose slowly with a delay and lasted much longer than the stimulation or drug application, unlike the faster transient effects recorded directly in C fibres (not shown). Furthermore, a blockade of GABA receptors (with 0.1μM L655708) prevented most of the C fibre mediated depolarization of afferents (see Fig 10E in Lucas-Osma et al. 2018), indicating that the indirect action of GABA on large diameter afferents causes the main depolarization in the dorsal root. Also, application of zolmitriptan to inactivate C fibres prevented the C fibre induced depolarization by icilin (Fig 2.4C) and by itself hyperpolarized the DRP (Fig 2.4F). The latter hyperpolarization of afferents

by zolmitriptan is important because it implies a spontaneous tonic C fibre-induced activation of afferents that plays an important role in sensory transmission. Interestingly, the large slow sensory axon depolarizations (DRPs; tonic PAD) induced by C fibre activation with icilin persisted in TTX (Fig 2.5A), suggesting that the proprioceptive afferents are depolarized by a local non-spiking circuit in the dorsal horn, as we have established previously (and sensitive to GABA blockers; see Fig 11 in Lucas et al. 2018).

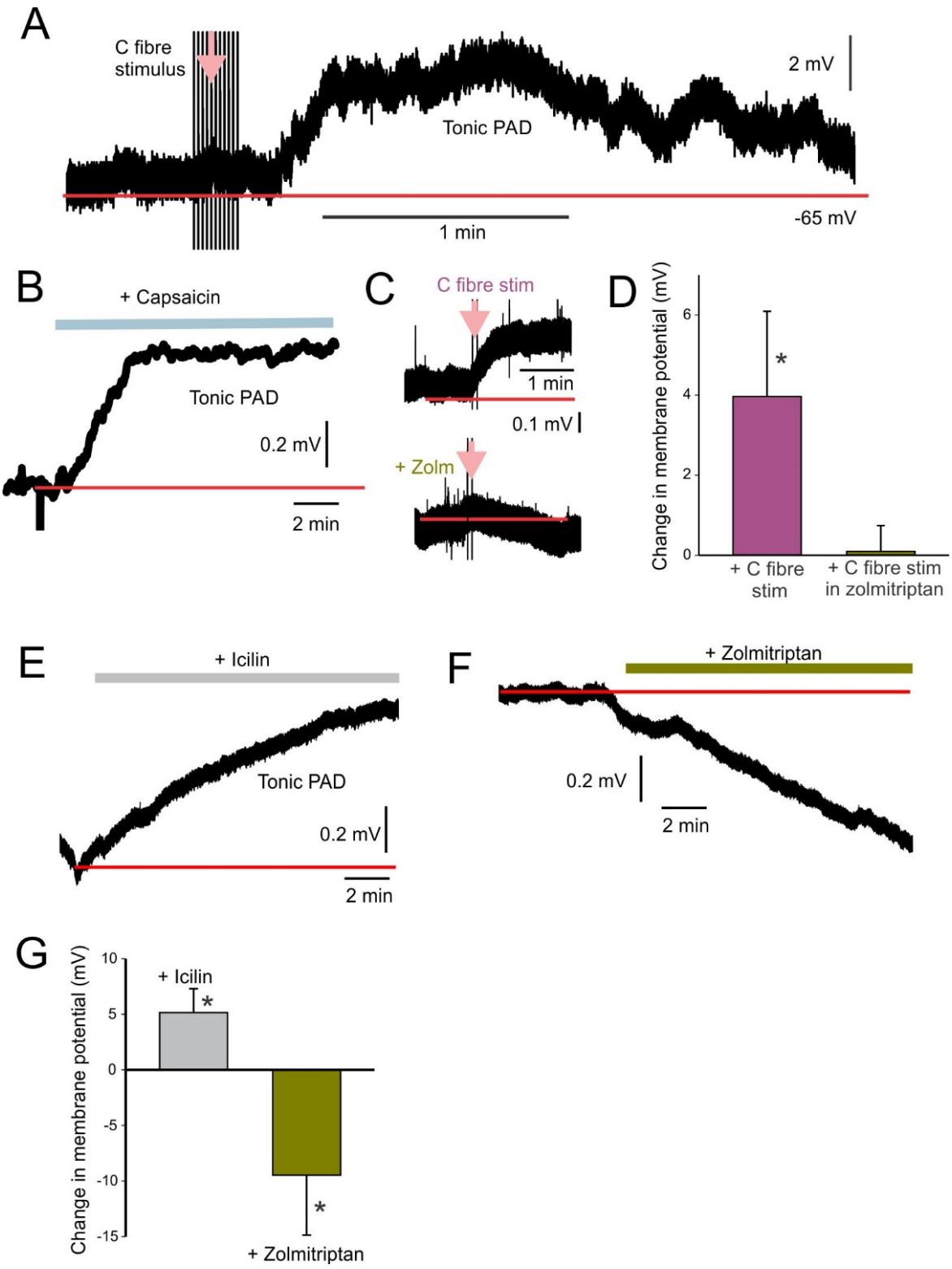


Figure 2.4. Activation of C fibres, both chemically and electrically, produces a tonic primary afferent depolarization (tonic PAD).

A: Intracellular recording from a proprioceptive group Ia afferent (S4 afferent) in the dorsal horn. Isolated C fibre stimulation (applied to dorsal root, 11 stims, 1 Hz, 50 x T) depolarized the Ia afferent membrane potential for several minutes (tonic PAD). **B**: Dorsal root potentials (compound PAD) recorded from the cut central end of a dorsal root (B-E). Capsaicin (10 μM) depolarized the afferent membrane potential for several minutes (tonic PAD, n = 3). **C**: Isolated C fibre stimulation of a dorsal root (11 stims, 1 Hz, 50 x T, dorsal root in low-dose TTX as detailed in methods) produced a depolarization of all primary afferents (n = 6), whereas C fibre stimulation in the presence of zolmitriptan did not cause any depolarization of the primary afferents (n = 5). **D**: Overall, C fibre stimulation caused afferent membrane depolarization (n = 6), whereas following the application of zolmitriptan (3 nM) this C fibre stimulation facilitation is eliminated (n = 6); shown as the change in membrane potential relative to control. **E**: Following the application of icilin (10 μM) the primary afferents depolarized for several minutes (tonic PAD) (n = 5). **F:** Intracellular recording from a proprioceptive Ia afferent (S4 afferent) in the dorsal horn. Zolmitriptan (3 nM) caused the Ia afferent membrane potential to hyperpolarize. **G**: Icilin caused afferent membrane depolarization (n = 6), whereas zolmitriptan caused afferent membrane hyperpolarization (n = 5); shown as the change in membrane potential relative to control. This suggests that not only do C fibres cause tonic PAD, but they must also be spontaneously active, steadily facilitating tonic PAD and associated sensory transmission (Fig 2.3). Error bars SD. * significantly different, $P < 0.05$.

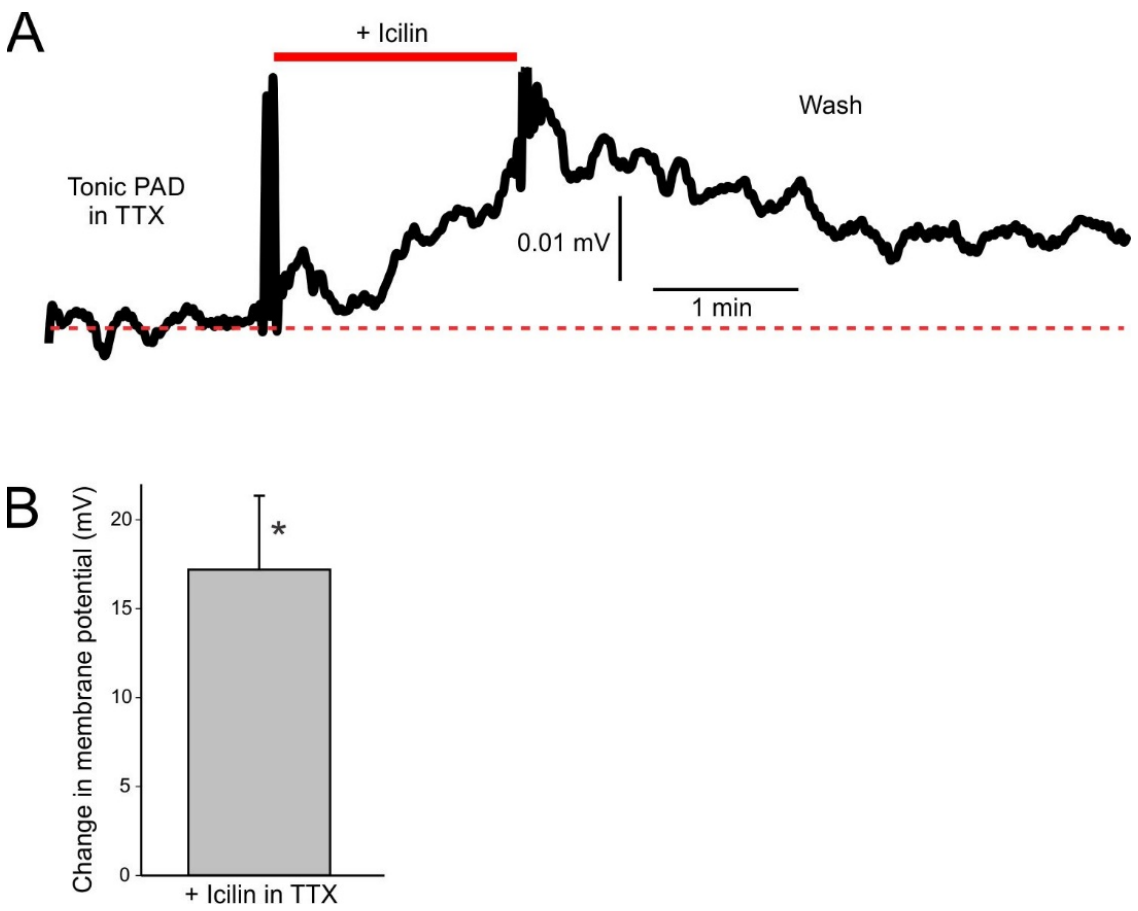


Figure 2.5. Activation of C fibres depolarizes the proprioceptive afferent membrane potential even when spike transmission is blocked by TTX.

A: C fibre activation with icilin (10 μM) depolarized the proprioceptive afferent membrane potential in the presence of TTX (2 μM) to block all spike transmission. When icilin is washed from the bath, this depolarization of the membrane potential ceased. **B**: Following the application of icilin to the spinal cord in the presence of TTX, the proprioceptive afferent membrane potential depolarized for several minutes indicating a tonic PAD (n = 5) shown as the change in membrane potential relative to control. This suggests that the proprioceptive afferents are depolarized by a local non-spiking circuit in the dorsal horn. Error bars SD. * significantly different, $P < 0.05$.

The mechanism by which this tonic PAD facilitates afferent transmission is still uncertain, but appears to involve facilitation of sodium spike conduction in the branch points of afferents, far from afferent terminals (Lucas-Osma et al. 2018), and this is consistent with our finding that C fibres affect proprioceptive afferents by local circuits in the dorsal horn, as just mentioned. That is, as already discussed, we have reported that this tonic PAD is mediated by α5 $GABA_A$ receptors and these receptors are mainly located at branch points near sodium channels on large proprioceptive afferents and surprisingly not at afferent terminals or boutons on motoneurons (Lucas-Osma et al. 2018). Because these α5 $GABA_A$ receptors are near sodium channel at branch points where conduction failure is possible (Walmsley et al. 1995), they may assist the spike. Indeed, we have previously found that they serve to depolarise nodes helping to prevent branch point failure (Lucas-Osma et al. 2018). Also, we have demonstrated that blocking α5 $GABA_A$ receptors with L655708 or bicuculline (inverse agonist of α5 $GABA_A$ receptors and competitive antagonist of $GABA_A$ receptors, respectively), hyperpolarized large proprioceptive afferents and accordingly decreased spike transmission (Lucas-Osma et al. 2018). Thus, ultimately tonic PAD increases the MSR by increasing sensory axon transmission.

This suggests the tonic PAD produced by C fibre stimulation (Fig 2.4A) should increase the MSR (Fig 2.2B-D) via preventing sodium channel branch point failure in large diameter proprioceptive afferents mentioned above. One way to further test this idea is to examine spike transmission on dorsal roots by examining antidromic spike transmission that often occurs in dorsal roots. That is, using both direct intracellular recordings from proprioceptive afferents, and extracellular dorsal root recordings, we measured antidromically propagating spikes that are known to occur when adjacent dorsal roots are stimulated. These spikes occur on the rising phase of the rapid phasic PAD evoked by low threshold large diameter sensory stimulation (2 x T) of

the adjacent dorsal root (we also at times stimulated and recorded from the same dorsal root; S3 or S4). We found that when we stimulated a dorsal root (1.1 x T, 0.1 ms) this evoked a large phasic PAD that produced an antidromic spike (action potential), as expected, but in some cases this action potential was not full height indicating that it failed to propagate antidromically past the recording electrode. However, when we stimulated C fibres (50 x T, 5 ms pulse) 20 s prior to the Ia stimulation, this improved the PAD-induced antidromic spike in the same afferent, with a full action potential propagating past the electrode (Fig 2.6B). We were able to record this failing spike because it induces a passive attenuated potential at a distance from the failure point (with smaller spikes indicating more distal failure; Fig 2.6C). Likewise, when we recorded from dorsal roots there were compound action potentials (termed dorsal root reflexes, DRRs) evoked by the underlying PAD (DRP; Fig 2.7A, D), and these were facilitated by the application of icilin (Fig 2.7D). Overall, this is consistent with the notion that C fibres facilitate a GABAergic PAD that helps prevent spike failure in axons.

In contrast, when we inactivated C fibres with zolmitriptan the antidromic spikes evoked by PAD (or directly evoked by ventral horn stimulation; Fig 2.8) were reduced (Fig 2.7B-E, DRR decreased; Fig 2.8B; volley reduced), even though the low threshold evoked phasic PAD did not change (Fig 2.7E). As mentioned in the introduction, phasic PAD is the classic PAD which is associated with presynaptic inhibition. Therefore, these results suggest that decreased C fibre activity (via zolmitriptan), reduces Ia afferent conduction, seen as reduced DRRs, whereas classic PAD and presynaptic inhibition play no role. The reduced DRRs likely occurred as a result of zolmitriptan hyperpolarizing the afferents (reducing tonic PAD) and brought some afferents below spike threshold, at least the spikes on the edge of failure.

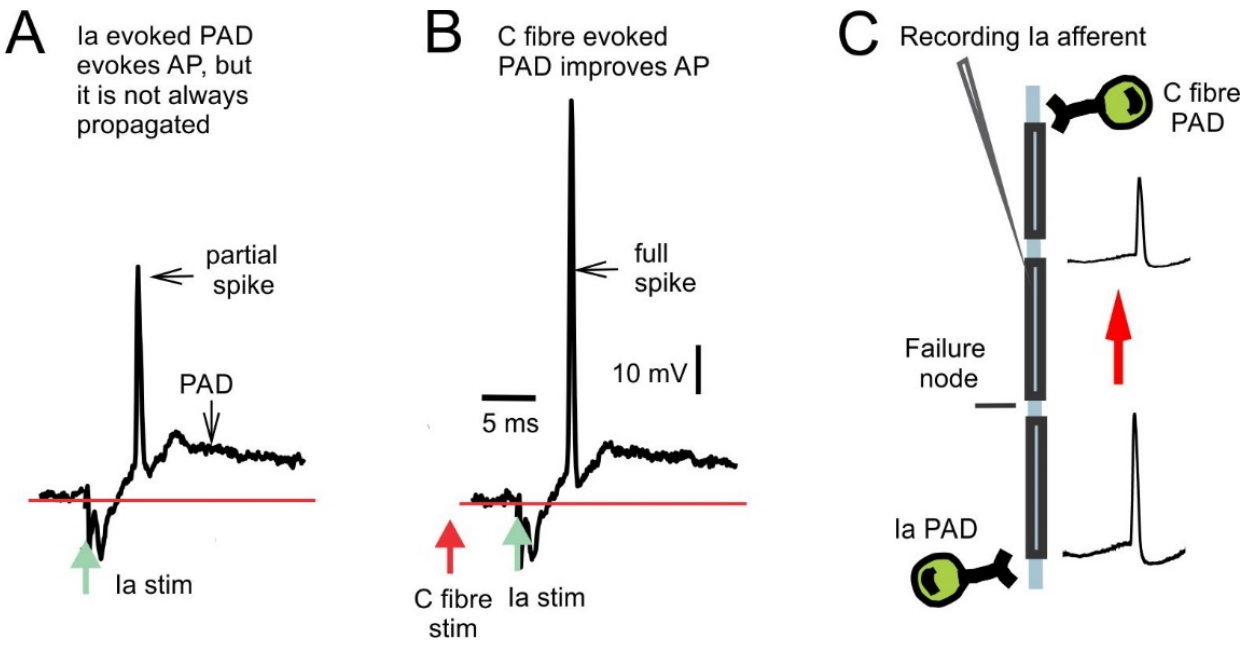


Figure 2.6. Activation of C fibres prevents failure of spikes in primary afferents.

A: Intracellular recording from a Ia afferent near the central canal. Ia stimulation (1.1 x T, 0.1 ms) evoked a large PAD that produced a spike on the rising phase of PAD, but in this case the action potential (AP; spike) was not full height, indicating that it failed to conduct antidromically past the electrode. **B**: C fibre stimulation (20 s prior, 50 x T, 5 ms pulse) depolarized the afferent (tonic PAD) and improved the PAD-induced antidromic spike in the same afferent, with a full action potential propagating by the electrode (n = 5; afferents similar). **C:** Schematic showing recording arrangement and suggested locations of GABAergic input (green cells) that produced PAD from a Ia afferent and C fibre stimulation. We were able to record the failing spike because it is an AP that has failed at a nearby node and therefore when the AP reached the recording electrode, we were still able to record a spike but it was reduced in size.

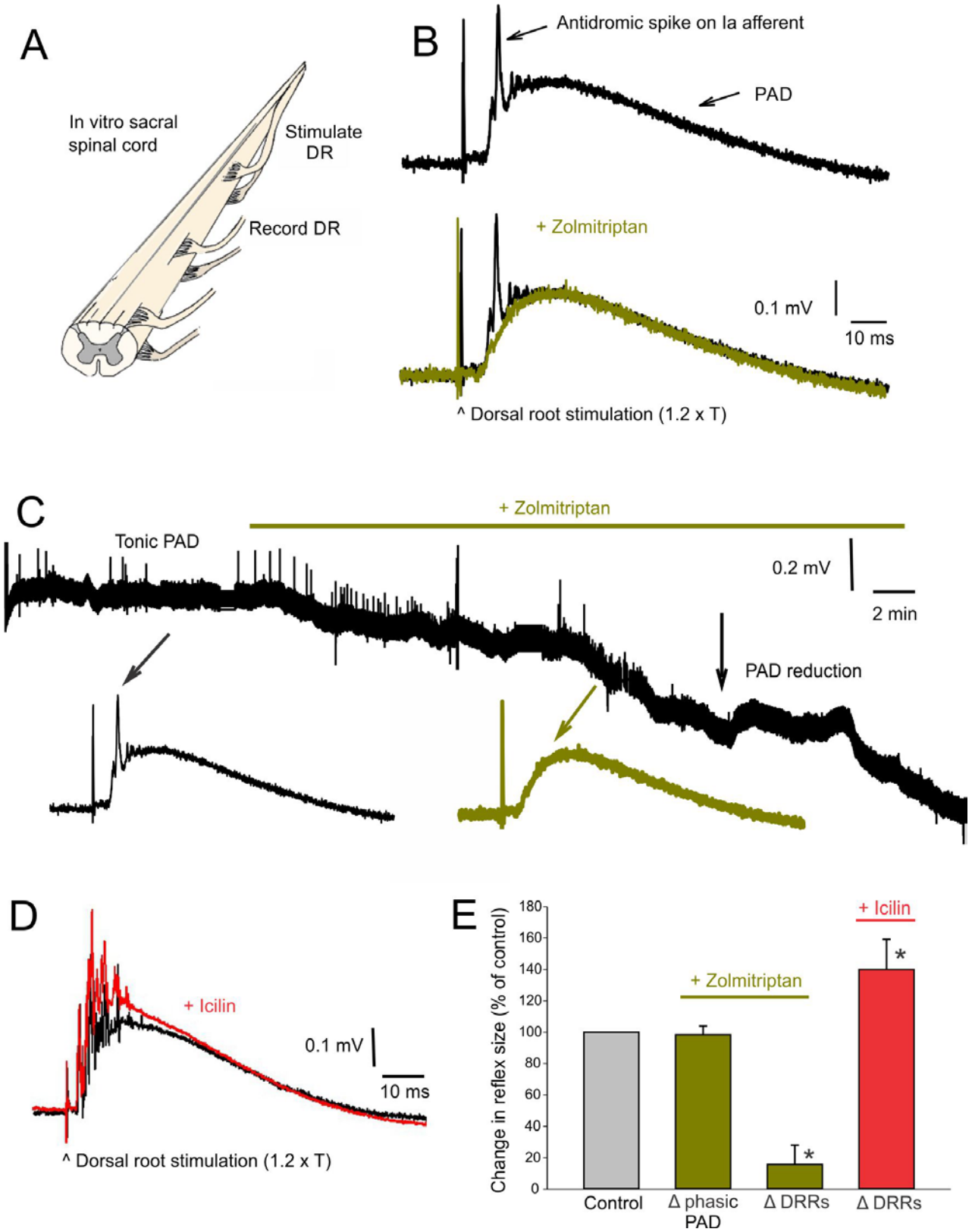

A
In vitro sacral spinal cord
Stimulate DR
Record DR
B
Antidromic spike on Ia afferent
PAD
+ Zolmitriptan
0.1 mV
10 ms
^ Dorsal root stimulation (1.2 x T)
C
+ Zolmitriptan
Tonic PAD
0.2 mV
2 min
PAD reduction
D
+ Icilin
0.1 mV
10 ms
^ Dorsal root stimulation (1.2 x T)
E
Change in reflex size (% of control)
180
160
140
120
100
80
60
40
20
0
+ Zolmitriptan
+ Icilin
*
*
Control
Δ phasic PAD
Δ DRRs
Δ DRRs

Figure 2.7. Inhibiting C fibres with zolmitriptan hyperpolarizes proprioceptive afferents and accordingly decreases spike transmission whereas activating C fibres with icilin has the opposite effect.

A: Schematic of setup to record PAD from the cut end of a dorsal root in response to stimulation of an adjacent dorsal root. **B**: Following low intensity dorsal root stimulation (2 x T, 0.1 ms pulse, group I stimulation) a phasic PAD occurred, lasting approximately 50 ms. This phasic PAD evoked antidromic afferent spikes (DRRs) on its rising phase (black trace). Zolmitriptan (10 nM) didn't change phasic PAD, but it reduced the antidromic spikes (DRRs), suggesting that decreasing spontaneous C fibre activity reduced afferent transmission (reduced DRRs), whereas classic PAD plays no role. **C**: Primary afferent membrane potential recorded from the cut end of a dorsal root. Zolmitriptan (10 nM) hyperpolarized the primary afferents for several minutes (top), and again blocked the DRR. This hyperpolarization likely blocked the antidromic spikes (DRR) occurring during the phasic PAD by bringing the membrane potential further from the spike threshold. This suggests that C fibre inactivation reduces C fibre-induced tonic PAD. **D**: Icilin (10 μM) increased the antidromic spikes (DRRs) indicating improved spike conduction. **E**: Following the application of zolmitriptan, phasic PAD didn't change (n = 8), however the DRRs decreased (n = 5). Icilin increased the DRRs (n = 5). This is shown as the change in phasic PAD and DRR amplitude relative to control (before zolmitriptan or icilin). This suggests that C fibres facilitate a tonic PAD that helps to prevent spike failure in axons. Error bars SD. * significantly different, $P < 0.05$.

A method to more directly assess proprioceptive afferent conduction is to selectively stimulate their terminals in the ventral horn and record the direct propagation of their spikes into the dorsal roots. Using a microelectrode placed in the S4 ventral horn we stimulated minimally to reduce current spread (at ~ 7 μA, 2 x T) and recorded the compound action potentials that managed to propagate antidromically to the dorsal root with our standard dorsal root recording arrangement, detailed in the methods. From the evoked compound action potential we assessed the earliest phase to avoid later slow afferents activated by current spread. In this way the amplitude of the compound action potential reflects the number of axons stimulated that did not fail to propagate spikes to the dorsal root. When we applied zolmitriptan to block C fibres, the compound action

potential recorded on the dorsal root was markedly reduced (Fig 2.8B-C), and importantly this action of zolmitriptan was blocked by inhibiting $GABA_A$ receptors with a pre-treatment of bicuculline (Fig 2.8C). This is consistent with our central conclusion that inhibition of C fibres reduces an endogenous tonic GABA tone (GABA-mediated tonic PAD), that normally facilitates afferent spike initiation and conduction. Indeed, we found that blocking $GABA_A$ receptors themselves with bicuculline mimicked the action of zolmitriptan (Fig 2.8D), again reducing the compound action potential, and again consistent with our central conclusion that $GABA_A$ activity is needed to help spike initiation and transmission.

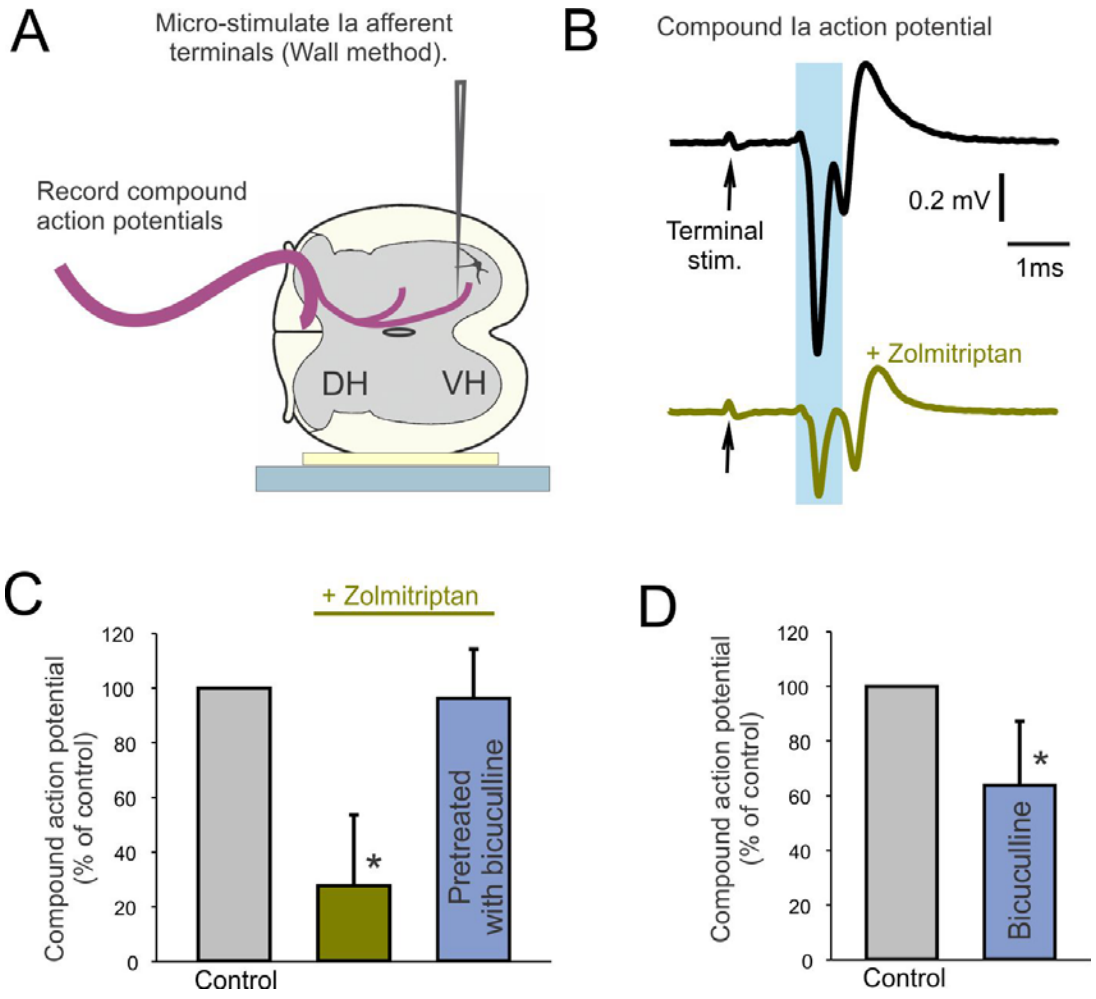


Figure 2.8. Inhibiting C fibres with zolmitriptan reduces sensory transmission to and from the ventral horn.

A: Micro-stimulation of Ia afferent terminals in the ventral horn in the isolated spinal cord (2 x T, 0.1 ms pulse), produced antidromic compound action potentials recorded from dorsal roots. **B**: Zolmitriptan (10 nM) reduces the Ia compound action potential, suggesting reduced spike transmission due to zolmitriptan hyperpolarizing the afferents and therefore reducing C fibre-induced tonic PAD, likely causing the afferents to be below spike threshold (n = 7). **C**: Zolmitriptan (3 nM) decreased the compound action potential (n = 7), but following pre-treatment with bicuculline (50 μM) to inhibit $GABA_A$ receptors, the compound action potential was no longer decreased following the application of zolmitriptan (n = 6), seen as a percentage of the compound action potential control. **D**: Application of bicuculline (50 μM) decreased the compound action potential, mimicking the action of zolmitriptan suggesting that $GABA_A$ receptor activity is required for spike initiation and transmission.

C fibres make direct contacts with GAD2 neurons

To determine how C fibres can activate the GABAergic neurons underlying PAD, we next examined the relation of dorsally located C fibres to GABAergic neurons. We immunolabelled C fibres with CGRP (Fig 2.9; grey) and their presynaptic terminals with Bassoon (Fig 2.9; white). GAD2 neurons, a subpopulation of GABAergic neurons, were genetically labelled in GAD2-cre-ER mice (Fig 2.9; blue). We found that C fibres directly synapse onto GAD2 neurons (Fig 2.9; white arrows, with Bassoon on C fibres presynaptic to the GABAergic neuron), establishing a possible anatomical basis for C fibre mediated PAD, though more indirect circuits cannot be ruled out, especially considering how long lasting C fibre actions are (Fig 2.2C).

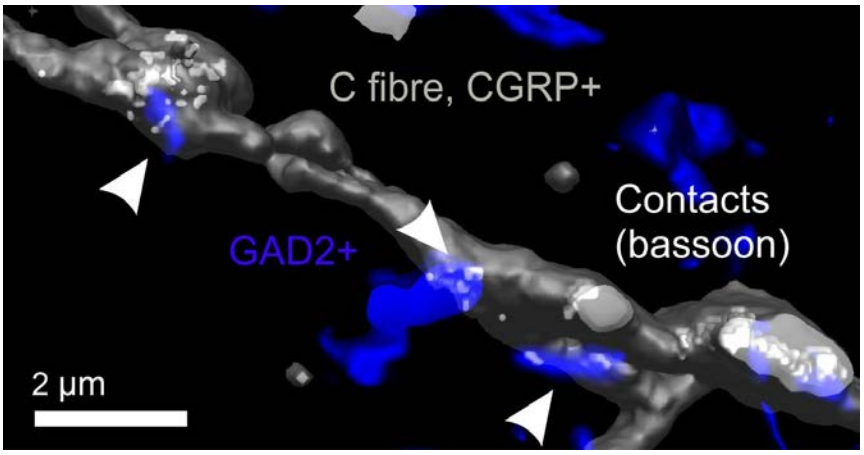


Figure 2.9 C fibres make direct contacts onto GAD2 neurons.

C fibres (immunolabelled with CGRP; grey) make direct contacts onto genetically labelled GABAergic GAD2 neurons (blue; in GAD2-cre-ER//flx-EYFP mice) in the dorsal horn (n = 5). Presynaptic contacts on C fibres are immunolabelled with Bassoon (white).

DISCUSSION

Our results demonstrate that C fibres facilitate spike transmission in large diameter proprioceptive afferents in the spinal cord, including orthodromic conduction from the dorsal roots to the ventral horn (Fig 2.3), and initiation and conduction of antidromic spikes starting in the spinal cord and propagating outward to the dorsal roots (Fig 2.6 and 2.7). This C fibre facilitation of afferent conduction directly leads to long lasting increases in monosynaptic EPSPs and reflexes in motoneurons, since we find that C fibre activity (as modulated by zolmitriptan) is not associated with long lasting postsynaptic changes in motoneuron excitability (Murray et al. 2010). This stands in sharp contrast to classical presynaptic inhibition of afferent transmission to motoneurons, thought to be mediated by GABAergic innervation of afferent terminals (Eccles et al. 1962; Rudomin and Schmidt. 1999), via GAD2 neurons (Fink et al. 2014; Hughes et al. 2005), and indeed we demonstrate that classical sensory evoked phasic PAD is relatively unaffected by altering C fibre activity (Fig 2.7B). Nevertheless, we also demonstrate that C fibre activity produces a tonic PAD that is dependent upon GABA, but via extrasynaptic $GABA_A$ receptors (Fig 2.4; Lucas-Osma et al. 2018), and this tonic PAD facilitates proprioceptive afferent spike transmission. Without this GABAergic tonic PAD, C fibres no longer facilitate afferent transmission (Fig 2.8C), and so the action of C fibres on sensory transmission must be indirect, acting via GABAergic neurons, as we discuss next.

The neuronal circuits that mediate C fibre facilitation of proprioceptive afferent sensory transmission are currently only poorly defined, though we know some critical elements. C fibres are known to directly innervate the many GABAergic neurons of the dorsal horn (Hughes et al. 2005; Fig 2.9), and in general GABAergic neurons provide the dorsal horn with a dense plexis of GABAergic terminals, with many more GABAergic terminals than in the ventral horn (Betley et

al. 2009; Fink et al. 2014; Hughes et al. 2005). We have also recently shown that large proprioceptive afferents have extrasynaptic GABA receptors along their whole length as they traverse through the dorsal horn on the way to the ventral horn, and it is these dorsally located GABA receptors that produce both tonic and phasic PAD (not terminal $GABA_A$ receptors; Lucas-Osma et al. 2018). Thus, it is reasonable to suppose that these dorsally located extrasynaptic GABA receptors on afferents are strongly activated by spillover of GABA from the dense GABA innervation of the spinal cord. Indeed, both Hounsgaard's group (Russo et al. 2000) and our group (Lucas-Osma et al. 2018) have shown that such spillover of GABA does indeed depolarize large afferents, and this is mediated via extrasynaptic GABA receptors (L655708 sensitive; Lucas-Osma et al. 2018). Furthermore, this GABA spillover and PAD is triggered by C fibre stimulation (Lucas-Osma et al. 2018). This can persist even when spikes are blocked in all but C fibres (which have TTX resistant sodium channels), and thus must at least in part be mediated by a local non-spiking circuit in the superficial dorsal laminae where the C fibres terminate (Lucas-Osma et al. 2018; Russo et al. 2000). Thus, the neuronal substrate by which C fibres facilitate large proprioceptive afferent transmission at a minimum involves a simple dorsally located circuit where C fibres activate GABAergic neurons which, in turn, activate extrasynaptic GABA receptors on these large afferents, though it likely also involves other GABAergic innervation of afferents along their whole length. Indeed, here we show that C fibres make direct contacts with GAD2 neurons, a unique genetically defined GABAergic neuron population (Fig 2.9). These GAD2 neurons are known to make small contacts with afferent terminals (Fink et al. 2014), however we believe that these GAD2 neurons make more extensive, larger contacts near afferent nodes, and are in the process of further detailing this (unpublished work). This would explain how C fibres can indirectly modulate tonic PAD. We

propose that C fibres make direct contacts with GAD2 neurons, which then synapse onto nodes on proprioceptive afferents, where we have previously shown extrasynaptic $GABA_A$ receptors modulate tonic PAD (Lucas-Osma et al. 2018).

The mechanism by which extrasynaptic $GABA_A$ receptors facilitate proprioceptive afferent conduction is also poorly understood, though it appears to be related to facilitation of sodium channels and associated spike initiation at the afferent nodes, because we find these $GABA_A$ receptors are mostly located at nodes, rather than at the afferent terminals (unlike what was previously supposed; Lucas-Osma et al. 2018). Further, we find that endogenous or exogenous activation of these nodal GABA receptors produces a tonic PAD that steadily facilitates spike initiation and conduction (Lucas-Osma et al. 2018). A clue to why this occurs is that both the nodes and $GABA_A$ receptors are almost always at one of the many branch points in the extensive arbor of the sensory afferents in the spinal cord (Lucas-Osma et al. 2018), and previously Wall and Henneman (Henneman et al. 1984; Wall and McMahon. 1994) have shown that branch point failure of axonal conduction is common in these large branching sensory afferents. Thus, we suggest that GABA assists spike transmission in large proprioceptive afferents by simply depolarizing the afferent nodes closer to spike threshold so they do not fail at branch points, as we have proven in preliminary studies (Li et al. 2017; Lucas-Osma et al. 2018) and are in the process of further detailing (unpublished work). Regardless of the final mechanism, at this point it is clear that C fibres activate nodal $GABA_A$ receptors that facilitate spike transmission. Critically, this C fibre mediated facilitation of sensory transmission is not via an additional non-GABAergic pathway, since blocking GABA transmission eliminates the modulation of spike transmission by C fibres (zolmitriptan no longer works, Fig 2.8).

The increased proprioceptive afferent conduction produced by C fibres has numerous important functional consequences, likely related to both sensory transmission to the brain and modulation of complex spinal motor circuits. We have detailed here the simplest of these functions, the MSR from afferents to motoneurons, which underlies the stretch reflex. Our results demonstrate that the increased sensory conduction produced by C fibres and GABAergic activity leads to increased EPSPs and MSRs on motoneurons. Perhaps the most surprising finding is that even spontaneous C fibre activity facilitates sensory transmission to motoneurons (increased MSR), since the MSR is reduced by reducing C fibre activity (with the 5-HT_{1D} agonist zolmitriptan) in the absence of exogenous activation of C fibres. Indirect in vivo measurements suggest similar conclusions, because 5-HT_{1D} agonists (zolmitriptan or sumatriptan) likewise inhibit the MSR in vivo (D'Amico et al. 2013; Honda et al. 2004). This suggests that C fibres are spontaneously active enough to contribute to a basal facilitation of sensory conduction. C fibres are peculiar because they express their sensory receptors (TRP receptors) all along their length in the spinal cord, as well as in the peripheral tissue they innervate. Thus, local events in the spinal cord (temperature, pH, etc.) contribute to overall C fibre activity, and combined with peripheral inputs, C fibres are known to exhibit a degree of spontaneous activity (Hulse et al. 2010; Pederson et al. 2005; Takahashi et al. 2011; Takashima et al. 2007). The TRPM8 receptor (which detects cold and menthol stimuli; activated by icilin) is particularly interesting in this regard because it is activated at room temperature (Andersson et al. 2004; Andersson et al. 2007), and thus in the periphery (in vivo) is spontaneously active. Thus, something as simple as skin temperature appears to modulate sensory transmission and reflexes in the spinal cord.

This present study was initially motivated by our recent finding that 5-HT_{1D} receptors are located exclusively on C fibres, and not alpha motoneurons or large afferents, even though they

strongly inhibit the MSR (Lucas-Osma et al. 2019). Thus, the question arose as to how these 5-HT receptors can modulate the MSR without being located on any element of the MSR pathway. We thus first explored the actions of C fibres on PAD, naively thinking it should increase PAD, since PAD has been classically associated with presynaptic inhibition of the MSR (Eccles et al. 1962; Rudomin and Schmidt. 1999; Wall. 1958). However, we found the opposite. C fibre activation produces a tonic PAD (Lucas-Osma et al. 2018). We now know that this tonic PAD facilities afferent conduction, rather the causing presynaptic inhibition. This solves the problem of how 5-HT_{1D} receptors function in the MSR: 5-HT_{1D} receptors inhibit C fibre activity which, in turn, reduces GABAergic neuron activity and extrasynaptic GABA receptor activity on proprioceptor afferent nodes, which in turn reduces spike conduction on the afferent (likely via an increase in branch point failure) and this ultimately reduces the MSR. This rather convoluted mechanism of the action of 5-HT_{1D} receptors likely explains some anomalies in the action of the 5-HT_{1D} agonist zolmitriptan, which have previously been reported. For example, after the application of zolmitriptan to the spinal cord, the resulting reduction in the MSR is difficult to reverse by washing zolmitriptan (Lucas-Osma et al. 2019), which likely involves the 5-HT_1 receptor somehow irreversibly reducing the spontaneous TRP receptor activity on the C fibres. This is plausible considering the intimate coupling of 5-HT_1 receptors and TRPM8 (Vinuela-Fernandez et al. 2014). Also, the finding that knockout of 5-HT_{1D} receptors leads to an increased MSR can now be viewed as mediated by possible increased C fibre activity.

In summary, we show the existence of a long lasting C fibre-induced tonic PAD which increases both orthodromic sensory transmission to the ventral horn and antidromic transmission. This occurs via a dorsally located microcircuit activating GABAergic neurons which, in turn, activate extrasynaptic $GABA_A$ receptors located at nodes, likely reducing branch point failure. When C

fibres are not active, this tonic PAD and associated facilitation of spike transmission ceases. Functionally, this C fibre and associated GABAergic activity leads to increased sensory transmission to motoneurons, and likely more generally leads to increased sensory transmission to the brain, as well as to complex spinal motor circuits. This C fibre mediated facilitation of non-noxious sensory transmission in large proprioceptive afferents stands in contrast to classic concepts of gating of sensory transmission (Melzack and Wall. 1965; Mendell. 2012; Moayedi and Davis. 2012), and thus warrants further study, especially as it relates to pain.

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Chapter 3: Thesis discussion and future directions

DISCUSSION

We have found C fibres activate GABAergic neurons (GAD2) that in turn produce a long-lasting depolarization of large diameter proprioceptive afferents (tonic PAD; Lucas-Osma et al. 2018), mediated by extrasynaptic $GABA_A$ receptors on these afferents. We find that this C fibre-induced tonic PAD assists with spike transmission. For example, it facilitates the propagation of afferent spikes (evoked directly or by phasic PAD) travelling antidromically to the dorsal roots. Additionally, it increases spike transmission travelling orthodromically to motoneurons in the ventral horn, seen as an increased MSR. We were able to corroborate these findings by inactivating C fibres using zolmitriptan and showing that this causes the afferent membrane potential to hyperpolarize, opposite to the depolarization (tonic PAD) which we observe with C fibre activation. This hyperpolarization likely places spikes below threshold and as such we observe reduced DRRs and a reduced MSR. Extrasynaptic α5 $GABA_A$ receptors located at branch point nodes mirroring the location of Na^+ channels, modulates tonic PAD which results from extrasynaptic GABA spillover (Lucas-Osma et al. 2018). It has been suggested that spike propagation to motoneurons routinely fails at the branch points mentioned above (Henneman et al. 1984; Walmsley et al. 1995), therefore logically tonic PAD modulated by extrasynaptic α5 $GABA_A$ receptors might aid in reducing this spike failure, resulting in the increased MSR which we observe. Given the strong tonic PAD we observe with C fibre stimulation it's likely that this results from spontaneous C fibre activity or perhaps spontaneous transmitter release from C fibres. This is reasonable to suggest as in our in vitro preparation TRPM8 receptors will be spontaneously active due to the temperature of the nCSF being maintained around 21°C resulting in the activation of these receptors (Andersson et al. 2004). Interestingly, we know that serotonin inhibits C fibre activity (Amrutkar et al. 2012; Zhao et al. 2016) and here we find that when we activate 5-HT_{1D} receptors, found exclusively on C fibres, with zolmitriptan, this significantly

hyperpolarizes C fibres, which in turn indirectly reduces tonic PAD (via reducing GABA) and thus results in afferent membrane hyperpolarization. We also observe reduced DRRs and a reduced MSR following the application of zolmitriptan. As such, here we show that we can modulate sensory transmission by reducing C fibre-induced tonic PAD. Since DRRs contribute to motoneuron reflexes and allodynia following nerve injury (Willis. 1999), as well as neurogenic inflammation and arthritis, and exaggerated MSRs contribute to adverse symptoms of spinal cord injury (SCI) such as hyperreflexia, our results shed some insight into how these undesirable symptoms could potentially be modulated by inhibiting C fibres with 5-HT. We use zolmitriptan in this thesis which is a medically viable drug to be used occasionally to treat migraine, but is not suitable to be used often due to its undesirable side effects such as nausea (Bird et al. 2014; González-Hernández et al. 2018). Our results demonstrate the potential for new drugs to be created or tested which activate 5-HT_{1D} receptors, or perhaps other 5-HT receptors, on C fibres. As such, modulating C fibre-induced tonic PAD could prove to have significant clinical utility.

Moreover, C fibres are known to make synaptic contacts with inhibitory neurons in the spinal dorsal horn (Peirs and Seal. 2016), but here we show for the first time that C fibres make direct contacts with GAD2 neurons (Fig 2.9). We know from our preliminary data (not shown) that when activated, these GAD2 neurons directly evoke a synaptic phasic PAD via a trisynaptic circuit involving group Ia proprioceptive afferents activating a first order excitatory neuron which, in turn, activates the GAD2 neurons which then contact back onto Ia proprioceptive afferents. We show that C fibres make direct contacts with GAD2 neurons (Fig 2.9), bypassing this trisynaptic circuit, thus demonstrating the disynaptic circuit involved in C fibre-induced tonic PAD (Fig 3.1). Additionally, in our preliminary data (not shown) we show these GAD2

neurons innervate the nodes of Ia afferents in the dorsal horn forming synapses and loose contacts that may provide extrasynaptic GABA to the extrasynaptic $GABA_A$ receptors which we know modulate tonic PAD (Lucas-Osma et al. 2018), and thus explains how C fibres indirectly produce tonic PAD.

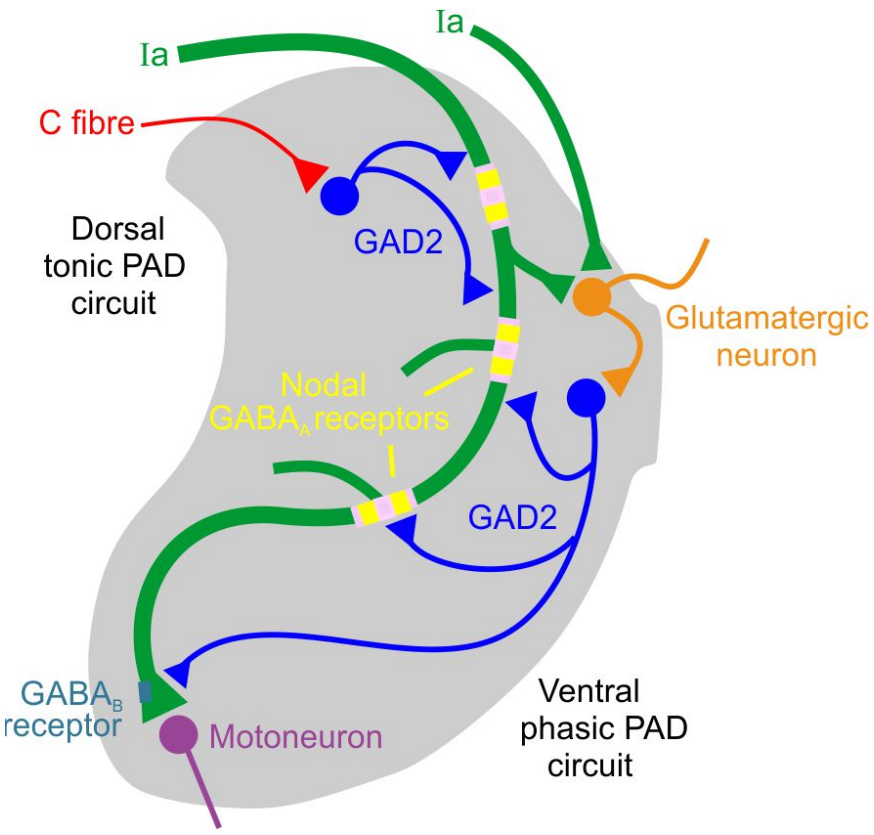

Figure 3.1 Proposed C fibre-induced tonic PAD circuitry within the spinal cord.

Classic trisynaptic loop of phasic PAD on right of diagram showing a proprioceptive Ia afferent (green) synapsing onto a glutamatergic neuron (orange) which synapses onto a GABAergic GAD2 neuron (blue) which, in turn, synapses back onto the Ia proprioceptive afferent (green). This Ia proprioceptive afferent projects to motoneurons (purple) in the ventral horn. Alternatively, GAD2 neurons (blue) can project directly to the afferent terminals on proprioceptive afferents, where we believe they may act on $GABA_B$ receptors (grey). On the left, C fibres (red) synapse onto GAD2 neurons (blue) which, in turn, synapse onto proprioceptive Ia afferents (green) in the dorsal horn, bypassing the classic trisynaptic loop of PAD, instead making disynaptic contacts. From our preliminary data (not shown), we see that dorsally located

GAD2 neurons (blue) innervate the nodes of Ia afferents (yellow), forming synapses and loose contacts which may provide extrasynaptic GABA to the extrasynaptic α5 $GABA_A$ receptors located at these nodes and which we know modulates tonic PAD.

FUTURE DIRECTIONS

Given our findings that there is a facilitatory C fibre-induced tonic PAD that facilitates spike transmission and is modulated by extrasynaptic $GABA_A$ receptors located at branch point nodes (Lucas-Osma et al. 2018), we have proposed that it is at these branch points where spikes are vulnerable to failure and tonic PAD serves to aid these failing spikes in their propagation by the actions of extrasynaptic α5 $GABA_A$ receptors mirroring Na^+ channels. As such, it is imperative to now investigate more extensively how often spikes are failing at these branch points and also to investigate the idea that GABA can be excitatory to afferent transmission, which is extremely counterintuitive to the traditional view of GABA. We will be able to confirm that spikes travelling into the spinal cord on dorsal roots often spontaneously fail to propagate into afferent branches, by using intracellular experiments to record from these fine afferent branches to measure failure after dorsal root stimulation. Then we can use nerve stimulation to activate PAD and assess whether this facilitates afferent spike transmission by bringing the axon closer to threshold.

Additionally, in order to assess the role of GABA, as mentioned previously, we have bred a strain of mice with GABAergic GAD2 neurons labelled with a fluorescent reporter, which can be activated optogenetically (Pinol et al. 2012). We are able to use these mice to optogenetically activate GAD2 neurons to show they evoke a synaptic PAD. We will now be able to confirm whether, as we suspect, GAD2 neurons can evoke a tonic PAD, confirming our proposed circuitry of C-fibre induced tonic PAD. Moreover, to study the role of axonal GABA tone, we

have a line of GAD2 mice which have been mutated to express the light-activated inhibitory archaerhodopsin Arch3 (Chow et al. 20120; Kralj et al. 2011), to selectively silence just dorsal GAD2 neurons, thus allowing us to investigate how sensory transmission is affected when there is a lack of dorsal GABA tone.

Given our findings relating to C fibre-induced tonic PAD and the effects activating 5-HT_{1D} receptors on C fibres has on this tonic PAD, it would be interesting to see if this C fibre-induced tonic PAD changes in a SCI model. We know that 5-HT inhibits the MSR via its actions on 5-HT_1 receptors including 5-HT_{1D} receptors (Honda et al. 2004; Honda et al. 2006; Lucas-Osma et al. 2019). Following SCI, there is a loss of brainstem derived 5-HT which contributes to muscle spasms and hyperreflexia due to a loss of inhibitory control resulting in exaggerated MSRs (Murray et al. 2010; Schmidt and Jordan. 2000). Here we find that reducing C fibre-induced tonic PAD using zolmitriptan reduces the MSR and zolmitriptan has already been shown to reduce sensory transmission in MSR and polysynaptic pathways, reducing long lasting reflexes (LRRs) (spasms) following SCI in human participants (D'Amico et al. 2013). Therefore it would be interesting to manipulate C fibre-induced tonic PAD in a rat model of SCI by activating C fibres using icilin and seeing if this increased LLRs, thus demonstrating that a possible mechanism modulating these LLRs could well be C-fibre induced tonic PAD.

The DRRs which we measured and have been implicated in several medical conditions outlined previously, also hold some promise for future research. That is, it would be interesting to use injury models of arthritis, neurogenic inflammation and allodynia, then manipulate C fibre-induced tonic PAD by either activating or inactivating C fibres using the methods we used, and seeing if these medical conditions are affected. This could potentially lead to a new approach and

new target for drugs to treat these medical conditions because we would have a greater understanding of the mechanism behind their etiologies.

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