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On the mechanisms of presynaptic inhibition of primary afferents

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Jacob Shreckengost

AB, University of Chicago, 2002

Advisor: Shawn Hochman, Ph.D.

An abstract of

A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University

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ABSTRACT

On the mechanisms of presynaptic inhibition of primary afferents

By

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Primary afferent neurotransmission is the fundamental first step in the central processing of sensory information and is controlled by pre- and postsynaptic inhibitory mechanisms. Presynaptic inhibition (PSI) is probably the more powerful form of inhibitory control in all primary afferent fibers. A major mechanism producing afferent PSI is via a channel-mediated depolarization of their intraspinal terminals, which can be recorded extracellularly as a dorsal root potential (DRP). Based on measures of DRP latency it has been inferred that this primary afferent depolarization (PAD) of low-threshold afferents is mediated by minimally trisynaptic pathways with pharmacologically identified GABAergic interneurons forming last-order axo-axonic synapses onto afferent terminals. This thesis describes recent and historical work that supports the existence of PAD occurring by more direct pathways and with a complex pharmacology that questions the proprietary role of GABA and $GABA_A$ receptors in this process. I show that cholinergic transmission contributes largely to PAD, including the possibility of direct release from primary afferents. I provide evidence for specific physiological actions of cholinergic receptors on distinct afferent class in modulating sensory transmission and discuss the possible role of cholinergic receptor activation in integration of sensory signaling both centrally and in the periphery. Specifically, cholinergic transmission underlies nicotinic receptor subunit specific actions on sensory gating of afferent subpopulations and plays a major role in generation of spiking in afferent axons, with the majority of the actions being on slower conducting low-threshold afferents. Finally, I describe unique expression patterns of nicotinic receptor subunits in defined populations of afferents. Together this work redefines how integration of sensory signaling occurs in primary afferents with implications for development of subunit specific pharmacological targets in the treatment of aberrant afferent signaling following disease or injury.

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Jesse, you're the reason that any of this was possible. Thank you and I love you.

CONTENTS

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LIST OF ABBREVIATIONS

PAD	primary afferent depolarization
DRP	dorsal root potential
DRR	dorsal root reflex
VRP	ventral root potential
EPSP	excitatory postsynaptic potential
EPSC	excitatory postsynaptic current
IPSP	excitatory postsynaptic current
EFP	extracellular field potential
AV	afferent volley
CAP	compound action potential
A-fibers	groups I and II muscle and groups Aβ and Aδ cutaneous afferents
C-fibers	groups III and IV muscle and group C cutaneous afferents
T	threshold: stimulus intensity required to recruit most excitable fibers
aCSF	artificial cerebral spinal fluid
GABA	γ-aminobutyric acid
ACh	acetylcholine
5-HT	serotonin or 5-hydroxytryptamine
NMDA	N-methyl-D-aspartate
nAChR	nicotinic acetylcholine receptor
$GABA_AR$	ionotropic $GABA_A$ receptor
Tib	tibial nerve
SP	superficial peroneal muscle
Su	sural nerve
PB	posterior biceps nerve
St	semitendinosus nerve

CHAPTER 1: INTRODUCTION

1.1 Overview of Presynaptic Inhibition

Synaptic efficacy can be modulated in a number of ways to influence signaling from the pre- to the post-synaptic cell. Pre- and post-synaptic mechanisms exist that employ ionotropic and metabotropic neurotransmitter receptors to influence either the excitability of the presynaptic terminal or the activity of the postsynaptic cell in response to presynaptic transmitter release. The studies below describe a novel form of ionotropic presynaptic inhibition in the rodent spinal cord and the transmitter systems involved. Here, I provide historical context for this work, some of the limitation of previous studies, and the need for a more refined approach using electrophysiological and pharmacological tools in combination with an elegant *in vitro* preparation to further dissect out the mechanisms of this phenomenon.

1.1.1 Presynaptic Inhibition in Invertebrates

Much of the understanding of the mechanisms and ionic basis of presynaptic inhibition (**PSI**) come from work in invertebrates. Using the crayfish neuromuscular junction as a model, which contains both excitatory and inhibitory motor neurons, Dudel and Kuffler (Dudel and Kuffler 1961) were able to show that even in cases where the inhibitory reversal potentials were more positive than excitatory junction potentials, simultaneous stimulation of the neurons resulted in a large *depression* of the excitatory junction potential. One would have expected that a positive going inhibitory potential combined with a positive going excitatory junction potential would have lead to an increased excitatory junction potential when nerves were stimulated simultaneously, not a decrease. This could only be explained by a presynaptic site of action with the inhibitory neuron forming an axo-axonic synapse onto the excitatory motor neuron innervating the muscle. In this conformation, stimulation of the inhibitory neuron leads to a

depolarization of the excitatory motor neuron, resulting in either a shunting of the action potential propagating down the excitatory neuron's axon or an inactivation of voltage-gated channels at the excitatory neuron's terminal, with both possibilities resulting in a decrease in transmitter release. A further study (Dudel and Kuffler 1961) on quantal size demonstrated that this inhibitory effect did not involve a decrease in quantal size as measured by miniature excitatory postsynaptic potentials resulting from release of a unitary quantum, but rather a decrease in the number of quanta that were released, demonstrating a decrease in excitability of the presynaptic terminal, further confirming a presynaptic site of action. The decrease in released quanta was attributed to an increase in membrane conductance of the presynaptic axon.

Investigation into the site of action on the presynaptic fiber as well as the mechanism of presynaptic inhibition led to studies that attempted to uncover if PSI resulted from conduction failure along the length of the axon or failure of the action potential to invade select branches of the axonal arbor. A block of action potential conduction would result from an increase in membrane conductance that acts to decrease the length constant of the axon. A decreased length constant would in turn decrease the length of propagation of an action potential along the unmyelinated terminals of an axon, resulting in a decreased depolarization at the axonal terminal and decreased neurotransmitter release. Support for failure of action potential conductance was provided by intra-axonal recordings in crayfish tactile afferents, which showed a decreased action potential size during primary afferent depolarization associated with PSI (Kennedy, Calabrese et al. 1974). It was then shown that the reduction in size of the axon potential could be caused by a depolarization of the axonal action potential, whereas injection of a hyperpolarizing current increased the size of the action potential (Dudel 1973). The increase in membrane conductance would also cause a shunting of action potential currents leading to conduction failure (Cattaert and El Manira 1999).

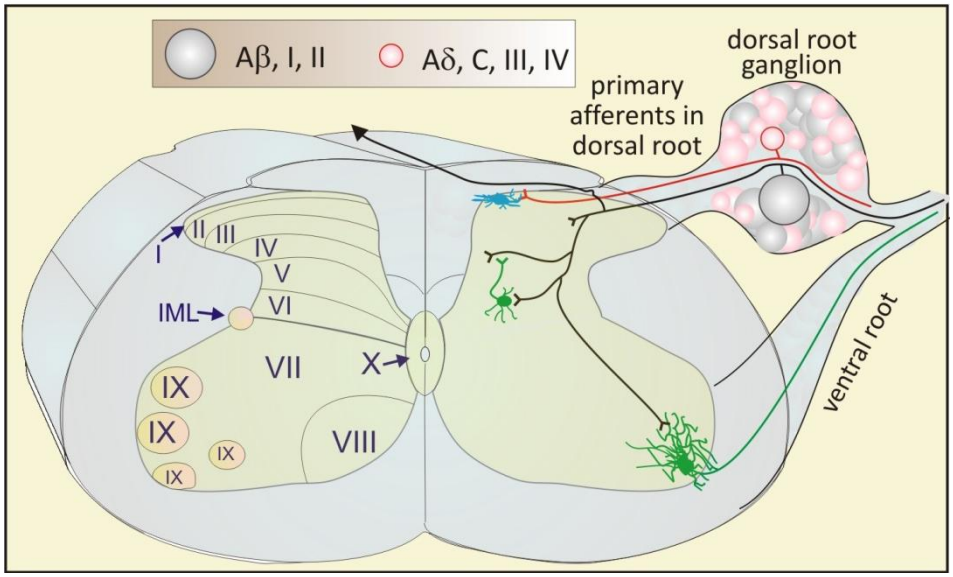
Further studies showed that PSI could be produced through diverse mechanisms. To determine if PSI could be due to conduction failure at axonal branch points, intra-axonal records of separate branches of a single nerve in the lobster were performed that demonstrated reduction, then block, of invading potentials at high frequency stimulation first in larger diameter axonal branches. This block of large diameter fiber invasion did not affect the smaller branches until much higher frequencies were used. If the sole mechanism of action were through an increase in extracellular potassium (Sykova 1981), leading to axonal depolarization and inactivation of sodium (Na^+) channels, then one would first expect to see block of small fibers, where the surface to volume ratio is the greatest before larger fibers (reviewed in (Nicoll and Alger 1979). Later studies showed that conduction block could be due to activation of γ-Aminobutyric $Acid_A$ (**$GABA_A$**) receptors, leading to an increase in chloride (Cl^-) conductance. However, application of GABA, while inhibiting the amplitude of end plate potentials, did not affect spontaneous release. This finding suggested that the receptors involved in PSI were not located directly at the terminals, but rather along the axons at more distant sites, such as branch points or at other locations along nerve axons (Takeuchi and Takeuchi 1966). This thesis provides further evidence that the receptors mediating depolarization of primary sensory afferent axons may, at least in part, be due to activation of receptors located along the length of the axon, not merely at the central terminals.

1.1.2Presynaptic Inhibition in Vertebrate Spinal Cord

The spinal cord is part of the CNS and is the interface for information transfer between body and brain. The constant stream of incoming sensory activity necessitates central mechanisms to limit and guide signaling. Sensory afferents enter the spinal cord through the dorsal roots, with the general organization of proprioceptive muscle afferents (Groups I & II) projecting to the motor pools in the ventral horn (lamina IX) and into ascending pathways to the brainstem for Group I and to deep dorsal horn (lamina V – VII) for Group II, and afferents encoding muscle temperature and pain information (Groups III and IV) synapsing mainly the in superficial dorsal horn (laminae

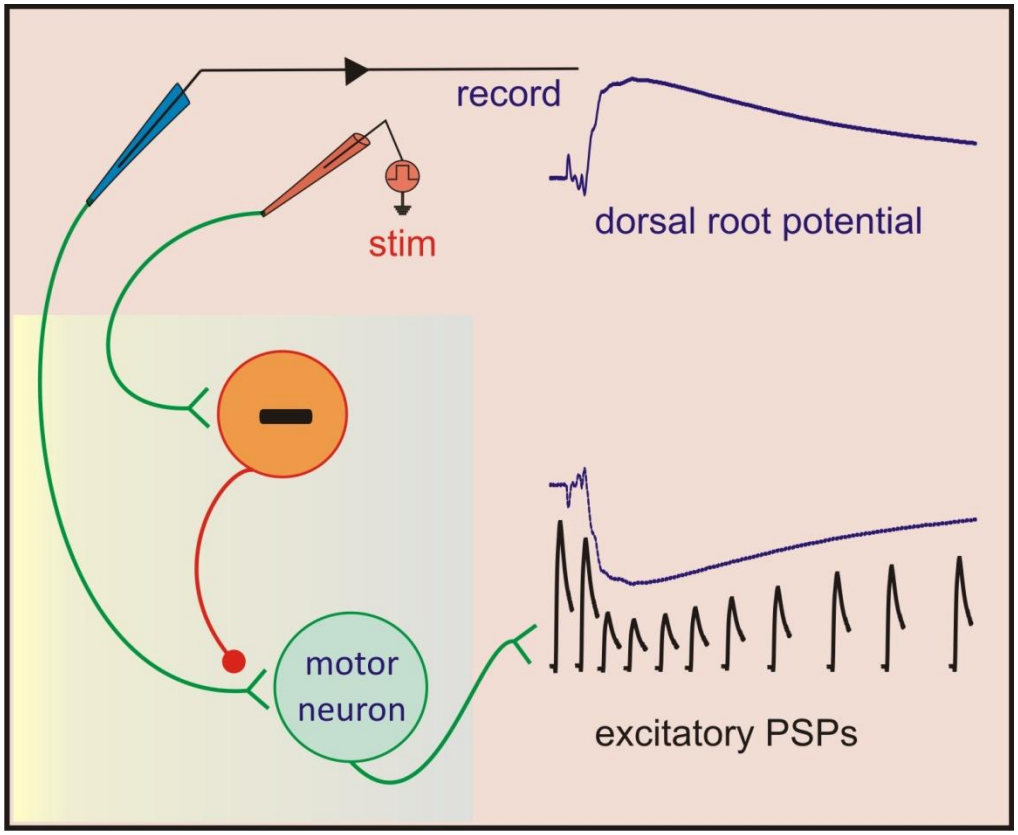
I & II). Cutaneous afferents have the majority of their synapses in the dorsal horn, with pain afferents (Groups Aδ & C) projecting mainly to the superficial laminae and non-pain afferents (Aβ) projecting to deeper dorsal horn areas (laminae V-VII; Figure 1). These different fiber types are all subject to synaptic integration when entering the spinal cord.

Figure 1 Spinal Cord Anatomy



Both presynaptic and postsynaptic inhibition regulate the effectiveness of sensory input onto central circuits (Solodkin, Jimenez et al. 1984; Rudomin, Solodkin et al. 1987). The intraspinal terminals of primary afferents represent the first CNS site for this control. A major mechanism producing PSI of primary afferents is via a channel-mediated depolarization of their intraspinal terminals (Rudomin and Schmidt 1999; Willis 1999), termed primary afferent depolarization (PAD) which paradoxically reduces transmitter release. This form of PSI is "more powerful than postsynaptic inhibition in depressing the central excitatory actions of almost all primary afferent fibers" (Eccles 1964). This occurs in cutaneous and muscle afferents and can be recorded experimentally following its antidromic electrotonic spread to dorsal roots as a dorsal root potential (DRP; Figure 2; for explanation see Figure 3). The DRP is typically viewed as a measure of PSI. This is based on intracellular recordings in motor neurons, which demonstrated an inhibition of motor neuron excitability that mirrored the DRP in time and duration without affecting the input resistance of the cell, suggesting a presynaptic site of action for the inhibitory mechanism (Figure 2). Based on measures of central latency, it has been assumed, though never directly demonstrated, that PAD is mediated by a trisynaptic pathway involving GABAergic interneurons (Rudomin and Schmidt 1999).

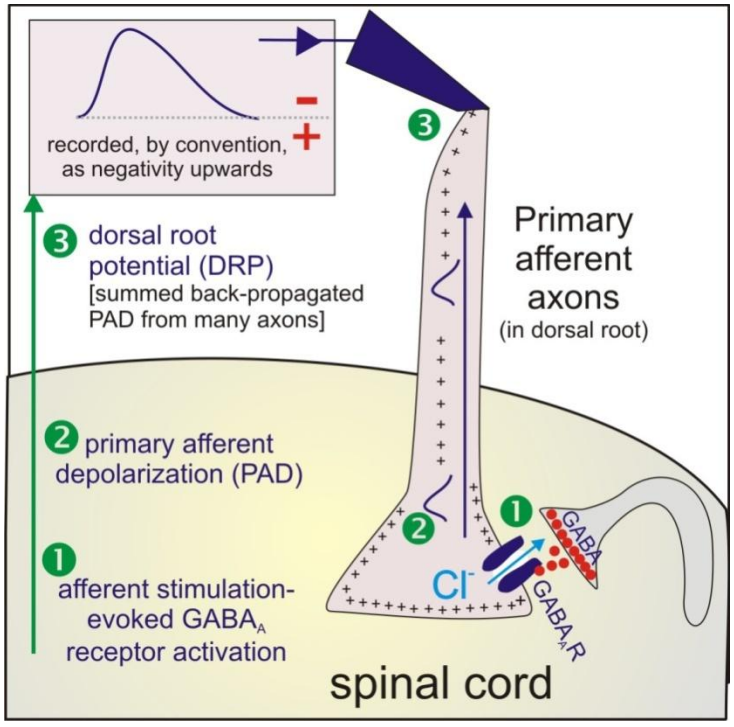
Figure 2 The DRP as a Measure of PSI



The DRP mirrors both the time course and amplitude of the inhibition of excitatory postsynaptic potentials in motor neurons. The DRP does so without changing the excitability of the motor neurons, thus suggesting a presynaptic site of action for inhibition.

The basic dogma of the mechanism of PSI consists of a trisynaptic pathway, where afferents synapse onto glutamatergic interneurons, which in turn activate second-order GABAergic interneurons that then form axo-axonic synapses onto primary afferent terminals (Figure 3). When $GABA_A$ receptors on primary afferent terminals are activated, chloride flows out of the terminals, due to the reversal potential of GABA in primary afferents being between -34 and -37mV (Deschenes, Feltz et al. 1976) . Using the $Na^+/K^+/Cl^-$ cotransporter, NKCC1, sensory neurons maintain a depolarizing chloride gradient. Hence, activation of bicuculline- and picrotoxin-sensitive intraspinal $GABA_A$ receptors located on $GABA_AR$ mediated PSI. Unlike most synapses, Cl^- depolarizes afferents and can be recorded as a dorsal root potential from sensory nerve roots. Sequence of events is numbered 1-3.primary afferents terminals results in PAD (Figure 3) (Eccles, Schmidt et al. 1963; Schmidt 1963; Barker and Nicoll 1973). Inactivation of voltage-gated Na^+/Ca^{2+} channels resulting in a decrease in intracellular Ca^{2+} accumulation in afferent terminals is generally thought to be one of the primary mechanisms through which PAD results in PSI (Kullmann, Ruiz et al. 2005). Another mechanism that could result in decreased Ca^{2+} entry at the terminal is through opening of voltage-gated Na^+ channels by PAD which then causes a current shunt when the action potential reaches the presynaptic terminal. Intracellular recording from motor neurons provide proof that the duration and amplitude correspond to the PSI of the monosynaptic reflex in motor neurons (Willis 1999). This inhibition of excitatory post-synaptic potentials (**EPSPs**) recorded from motor neurons is not accompanied by a change in conductance of the motor neurons themselves, pointing to a presynaptic site of action. Taken together, these findings show the DRP to be an ideal measure of PSI in primary afferents.

Figure 3 Production of the DRP



Presynaptic inhibition of primary afferents can be activated by afferents of the same or differing phenotype, descending systems, or spinal circuits typically thought to act via $GABA_A$ergic pathways (for review see (Rudomin and Schmidt 1999; Rudomin 2009)). The power of PAD in generating PSI is such that afferents entering a given segment of the spinal cord, when activated, are able to produce DRPs in afferents along the length of the entire cord (Lidierth and Wall 1998).

Presynaptic inhibition can occur at the spinal terminals of afferents before the first synapse in the spinal cord or at branch points on afferent arborization to block action potential invasion (Howland, Lettvin et al. 1955; Verdier, Lund et al. 2003). Presynaptic inhibition can also be highly selective by inhibiting specific intraspinal terminals without affecting other branches of the same afferent (Eguibar, Quevedo et al. 1994; Eguibar, Quevedo et al. 1997). Thus PSI is able to regulate sensory inflow such that no spinal neurons receive information from the inhibited afferents. Additionally, PSI exerts longer lasting effects, reducing reflex effectiveness for hundreds of milliseconds (300-400 ms) while postsynaptic inhibition lasts only tens of milliseconds (~10-30 ms) (Eccles, Schmidt et al. 1962; Gossard and Rossignol 1990). In contrast, postsynaptic inhibition alters responses to *all* inputs to a postsynaptic cell by changing the postsynaptic cell's excitability. Presynaptic inhibition clearly offers a highly efficient, selective, and effective mechanism for sensory regulation.

1.1.2.1 Presynaptic Inhibition Muscle Afferents

Presynaptic inhibition is seen in both muscle and cutaneous afferents. The anatomical basis for PAD mediated PSI is thought to be partially due to axo-axonic synapses onto primary afferents. Histological studies on Ia boutons (afferents bringing muscle stretch and velocity information from muscle spindles) in the ventral horn showed that 86% of the boutons investigated contained at least one axo-axonic synapse (Pierce and Mendell 1993). There is a differential distribution of axo-axonic contacts on different branches of Ia afferents, with the greatest concentration in

lamina IX (the motor nucleus of the ventral horn) (Walmsley, Graham et al. 1995), suggesting a differential control of different axonal branches. Branch control at single afferents is also supported by electrophysiological studies (Eguibar, Quevedo et al. 1997; Quevedo, Eguibar et al. 1997).

Ib afferents (arising from Golgi tendon organs) show similar synaptic contacts. Reconstruction of two axon collaterals identified by stimulation and injection with rhodamine dextran showed 59 axo-axonic contacts, with a differential distribution along the axonal branches. This reconstruction was further confirmation of the specificity of PSI in primary afferents and of the exquisite control of afferent inflow into the spinal cord by PSI ((D'Incamps, Destombes et al. 1998) reviewed in (Rudomin and Schmidt 1999)). Finally, functionally identified group II afferents (slowly and non-adapting afferents from muscle spindles communicating stretch information) labeled with horseradish peroxidase staining contained many axo-axonic contacts. Functional identification of group II afferents often revealed more than one synapse for each afferent axon (Maxwell, Kerr et al. 1997). Additionally, group II afferents are often identified in triadic arrangements with interneurons synapsing both on presynaptic Group II afferent terminals and on the postsynaptic targets of those same afferents. Many of the group II presynaptic terminals are positive for GABA and a portion of those are also positive for glycine labeling.

Modality specific patterns of PAD are evidenced by afferent subpopulations showing preferential actions only at defined modalities of afferents. Ia afferent fibers are depolarized by volleys in Ia and Ib fibers preferentially in flexors, but not extensors. In contrast, group Ib afferents are equally depolarized by volleys in other Ib afferents to both flexors and extensors. High threshold muscle and cutaneous afferents have little effect on either group Ia or group Ib. Group II afferents are most effectively depolarized by group II - again preferentially to flexors - cutaneous and articular afferents. They are also depolarized by iontophoretic application of the monoamines, dopamine (**DA**), serotonin (**5-HT**), and norepinephrine (**NE**) (Bras, Cavallari et al.

1989). A full description of the selectivity of afferents giving versus afferents receiving PSI is reviewed by Willis (Willis 2006).

1.1.2.2 Presynaptic Inhibition in Cutaneous Afferents

Cutaneous afferents are most strongly depolarized by other cutaneous afferents. Low-threshold cutaneous afferents also receive PAD from Ib, group II and group III afferents (Eccles, Schmidt et al. 1963).While there is evidence for PAD in large cutaneous afferents due to $GABA_A$ receptor activation (Jimenez, Rudomin et al. 1987), the case is less clear with high-threshold cutaneous afferents. Histochemical studies investigating primary afferent terminals in laminae II and III of the dorsal horn, where several modalities of cutaneous afferents terminate, have shown that less than 1-4% of all synapses observed are axo-axonic (reviewed by (Alvarez 1998)). Interestingly, these high threshold cutaneous afferents show a marked increase in antidromic discharges, or dorsal root reflexes (DRRs), during inflammation due to an increase in PAD (Willis 1999). This has led investigators to hypothesize that extrasynaptic GABAergic mechanisms may be responsible for the increase in DRRs seen in high-threshold cutaneous afferents after inflammation. However, this thesis will show evidence that extrasynaptic nicotinic acetylcholine receptors located along afferent axons are able to increase dorsal root spiking and that activation of nicotinic acetylcholine receptors are involved in the production of DRRs, suggesting that nicotinic mechanisms, independent of $GABA_A$Rs are responsible for DRRs in primary afferents.

1.1.2.3 Neurotransmitter Systems Implicated in Primary Afferent Depolarization

Several neurotransmitter systems have been implicated in PSI of various afferent modalities. While GABA has historically received the lion's share of investigators' attentions, the actions of glutamate, 5-HT, and ACh on their respective presynaptic receptors have also been described in relation to modulation of transmitter release from primary afferents, and these findings are summarized below.

1.1.2.3.1. $GABA_A$

$GABA_A$ receptor-mediated PAD has been the most extensively studied of any of the transmitter systems implicated in ionotropic PSI. Histologically, nearly all of the identified axo-axonic synapses identified on Ia and group II afferents in the motor nucleus have been positive for GABA immunoreactivity in cat, rat, and monkey, mostly located at or near the terminals of the primary afferent axons. Interestingly, most of the group II afferent terminals were contacted by afferents that showed co-localization of both GABA and glycine. Similarly, Ib afferents, like Ia afferents, show GABAergic presynaptic contacts, though this subtype has not been examined in as much detail. In contrast, cutaneous afferents do not have as many axo-axonic synapses present on their terminals. However, those cutaneous afferents examined in lamina II did show GABAergic presynaptic contacts (reviewed in (Alvarez 1998; Rudomin and Schmidt 1999).

Pharmacological studies have also shown that DRPs are reduced by the $GABA_AR$ antagonists bicuculline and picrotoxin (Eccles, Schmidt et al. 1963). Further support for GABAergic involvement in PSI comes from the ability of $GABA_AR$ agonists and modulators to produce PAD (Schmidt 1963; Barker and Nicoll 1973; Desarmenien, Santangelo et al. 1984). In Chapter 2, I address the complications that arise from interpretation of these results due to overlapping pharmacology of the Cys-loop ligand gated family of receptors, to which $GABA_A$, glycine, 5-HT and nicotinic AChRs, belong. Finally, in Chapter 5, I will address $GABA_A$-mediated PAD in comparison to nicotinic AChR-mediated PAD and demonstrate that they are separate phenomena with likely differing physiological roles.

1.1.2.3.2. Glutamate

Axo-axonic synapses are found in abundance on unmyelinated fibers (Huettner 1990; Liu, Mantyh et al. 1997). Selective depolarization of C-fibers is seen upon exposure of these fibers to the ionotropic glutamate receptor agonist, kainate. As such, presynaptic 2-amino-3-(5-methyl-3-oxo-1, 2- oxazol-4-yl) propanoic acid (AMPA), kainite and N-methyl-D-aspartate receptors

(NMDARs) have been proposed to modulate glutamate release from the central terminals of primary afferents. Indeed, presynaptic NMDARs on both low- and high-threshold fibers have been shown to play a role in inhibition of glutamate release from primary afferents in the rodent spinal cord through PAD(Bardoni, Torsney et al. 2004). Electron microscopy studies show the presence of these receptors on both myelinated and unmyelinated fibers (Coggeshall and Carlton 1998). Due to the cation-conducting nature of the receptors, they may modulate release through terminal depolarization and Ca^{2+}entry into the presynaptic terminal (Engelman and MacDermott 2004). Based on activation profiles and receptor kinetics in other brain areas, presynaptic receptors on primary afferents may also be activated by glutamate spillover, acting as autoreceptor-mediated negative feedback control (summarized in (Engelman and MacDermott 2004). Finally, there is evidence for a non-traditional mechanism of presynaptic glutamate receptor activation. For example, astrocytes have also been shown to modulate the probability of release by release of glutamate, suggesting another potential presynaptic site of action (Fellin, Sul et al. 2006).

1.1.2.3.1. Acetylcholine: Nicotinic

Primary afferents contain numerous nicotinic receptor subtypes (Genzen, Van Cleve et al. 2001; Lips, Pfeil et al. 2002). The presence and distribution of various nicotinic receptors subtypes in primary afferents will be described in detail in Chapter 5. Though PSI is thought to be largely mediated by activation of $GABA_A$Rs on primary afferent terminals, much of this is based on the ability of the $GABA_A$R antagonists, bicuculline and picrotoxin, to block the afferent-evoked DRP. However, bicuculline (IC_{50} = 0.8μM) potently inhibits the α9 nicotinic ACh receptor subunits (Rothlin, Katz et al. 1999), which are capable of forming homomeric receptors and are expressed in primary afferents (Lips, Pfeil et al. 2002). nAChR α10 subunits are also sensitive to bicuculline and strychnine (Alexander, Mathie et al. 2007). The subunits α9 and α10, which can form heteromeric receptors, are co-expressed in DRG (Lips, Pfeil et al. 2002). This raises the

possibility that a significant part of PAD is nicotinic receptor-mediated. Primarily, larger diameter (i.e. low-threshold) DRG neurons contain α-bungarotoxin binding sites (examined in rat, cat, monkey, and human) (Ninkovic and Hunt 1983). These neurons represent at least 12% of large diameter afferents and appear to project selectively to lamina III, the projection site of Aβ cutaneous afferents (Willis 1991) and presumably the location of interneurons interposed in PAD of cutaneous afferents (Jankowska, McCrea et al. 1981). Lamina III is also a location of dorsal horn cholinergic interneurons, which receive input from both myelinated and unmyelinated axons (Olave, Puri et al. 2002). It is important to note that 25% of presynaptic synapses onto primary afferents in laminae II and III contain ACh (Ribeiro-da-Silva and Cuello 1990). Furthermore, after dorsal rhizotomy, α-bungarotoxin binding *specifically* decreases in lamina III, and *nowhere* else in the spinal cord (Ninkovic and Hunt 1983), suggesting that the primary afferents projecting here are expressing ACh receptors. Thus, dorsal horn cholinergic neurons are well positioned to produce PAD (Ribeiro-da-Silva and Cuello 1990; Todd 1991; Alvarez 1998). Additionally, ACh has been shown to directly depolarize primary afferents in frog and rat, pointing again to its role in producing PAD (Shand 1965; Barker, Nicoll et al. 1975; Nicoll 1975).

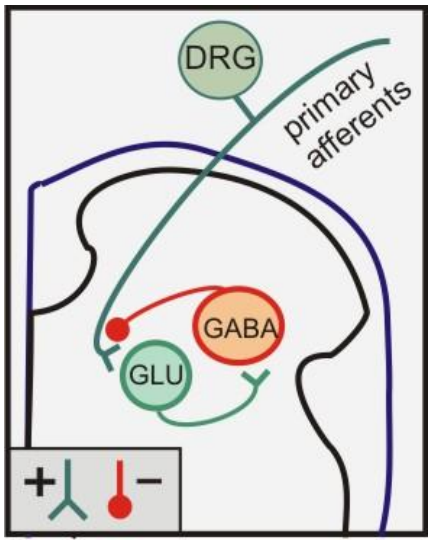
1.1.2.3.1. Serotonin: 5-HT$_3$

The ionotropic 5-HT$_3$ receptor has also been implicated in PSI of primary afferents. In particular, Khasabov et al showed that 5-HT$_3$ agonists depressed EPSPs in dorsal horn neurons of the young rat following stimulation of primary afferents at intensities sufficient to recruit all afferent fiber types (Khasabov, Lopez-Garcia et al. 1999). Furthermore, *in situ* hybridization showed a loss of 5-HT$_3$ binding, particularly in the superficial dorsal horn following unilateral rhizotomy (Kia, Miquel et al. 1995). A similar loss of 5-HT$_3$binding was seen after capsaicin-induced ablation of small unmyelinated afferents (Hamon, Gallissot et al. 1989). Combined, these studies suggest 5-HT$_3$ receptor involvement in PSI of small unmyelinated nociceptive afferents.

1.1.2.4 Limitations of Previous Presynaptic Inhibition Studies

Based largely on measures of central latency, it has been assumed, though never directly demonstrated, that PAD of low-threshold, large diameter afferents is mediated by a trisynaptic pathway involving last-order GABAergic interneurons acting on $GABA_A$ receptors(Figure 4) (Nicoll and Alger 1979; Rudomin and Schmidt 1999). Yet, to this very day, PAD-producing interneurons have never been unambiguously identified. So, although it is assumed that anatomically-identified GABAergic axo-axonic synapses onto primary afferents arise from the GABAergic interneurons mediating PAD, there continues to be no direct electrophysiological evidence. Additionally, for PAD of low-threshold afferents, only 22 interneurons have so far been identified (based on extracellularly recorded action potentials being followed by PAD) and only eight have latencies suggesting a last-order and hence GABAergic identity (Rudomin, Solodkin et al. 1987; Jankowska 1992; Jankowska and Riddell 1995). Of these eight, the discharge of the interneuron occasionally follows the onset of PAD in the primary afferents instead of preceding it, suggesting that firing in these interneurons is merely correlative, not causative. Based on these observations, Wall stated: "I believe we should remain cautious in the identification of those nerve cells which lead singly or in a chain to the generation of PAD (Wall 1998)."

Figure 4 Assumed Trisynaptic Pathway Mediating PAD



It has been assumed the DRPs are produced by a minimally trisynaptic pathway, depicted here. Primary afferents activate glutamatergic interneurons that then excite GABAergic last-order interneurons. These last-order GABAergic interneurons form axo-axonic synapses on primary afferent terminals to mediate PSI, as outlined in Figure 3.

If PAD occurred via a trisynaptic pathway, it should be reduced by anesthetics like pentobarbital, which decrease excitatory synaptic activity in all neurons (Franks and Lieb 1994). Most of the studies of PAD by Rudomin, Eccles, Jankowska and their colleagues used pentobarbital as the anesthetic in the decerebrate or spinalized cat (Eccles, Kostyuk et al. 1962; Jankowska, McCrea et al. 1981; Jimenez, Rudomin et al. 1984). As stated by Eccles et al (Eccles, Schmidt et al. 1963), *"The cutaneous volleys produce DRPs at such deep anaesthesia that their occurrence in the absence of all interneuronal activity has been suggested (Wall 1958). If this were established it would falsify the hypothesis that presynaptic inhibition is due to […] activation of specific interneuronal pathways."* While barbiturates could possibly strengthen PAD by directly potentiating $GABA_A$ergic transmission (Franks and Lieb 1994; Willis 1999), interneurons must be activated in a trisynaptic pathway. It is now known that pentobarbital potently inhibits glutamatergic transmission (AMPA receptor: IC_{50} = 50μM ≈ 1mg/kg) (Marszalec and Narahashi 1993; Jackson, Joo et al. 2003). Moreover, the lethal dose of 64mg/kg (~3.2mM) used by Eccles (Eccles, Schmidt et al. 1963) to finally block PAD would do so by an open channel block of the $GABA_A$ ionophore (IC_{50} = 3.2 mM).

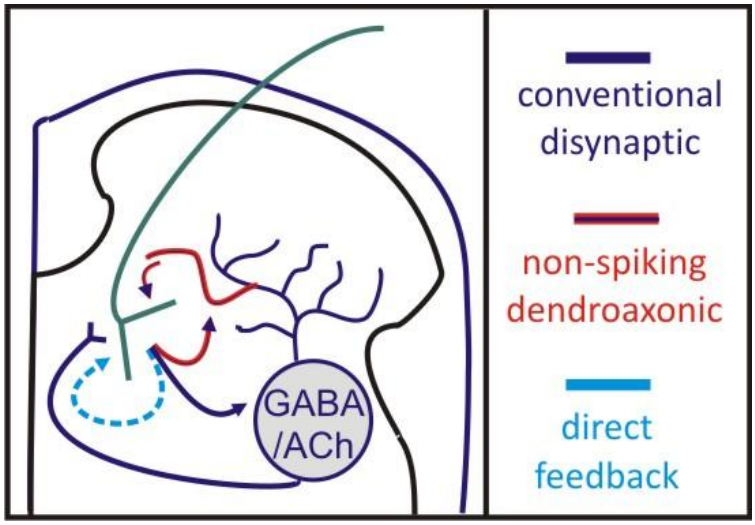
1.1.2.5 Other Possible Mechanisms Generating Afferent-Evoked PAD.

Other mechanisms of action can potentially evoke PAD. One possible mechanism proposed for PAD in pain fibers is dendritic release of transmitter in a non-spiking microcircuit (Figure 5) (Russo, Delgado-Lezama et al. 2000). Alternatively, PAD could be directly evoked via homosynaptic or heterosynaptic negative feedback of transmitter released from primary afferents (Figure 5). Certainly, this is the most common form of PSI found for CNS neurons (Engelman

and MacDermott 2004). However, afferent-evoked PAD lasts hundreds of milliseconds (Rudomin and Schmidt 1999; Willis 1999) and it is difficult to reconcile how such a brief synaptic input leads to a prolonged output.

In the absence of interneuronal action, prolonged PAD may be due to slow dynamics of transmitter release/uptake (Nicoll and Alger 1979). An unexplored mechanism of primary afferent PSI involves spillover to extrasynaptic receptors (Kullmann, Ruiz et al. 2005). For example, GABA spillover modulates the excitability of hippocampal mossy fibers (Kontro, Marnela et al. 1980) by acting at autoreceptors. Similar spillover mechanisms in primary afferents may also explain how afferent fibers from one population can occasionally produce PAD on other afferent populations (Figure 5) (Eccles 1963; Eccles 1963; Eccles 1963; Eccles, Schmidt et al. 1963). Miller and Woolf (Miller and Woolf 1996) identified long-lasting ionotropic glutamatergic excitatory postsynaptic currents that showed a slow decay of synaptic currents from high-threshold primary afferents. These glutamatergic currents had a time constant of seconds, were blocked by 6-cyano-7-nitroquinoxaline-2, 3-dione (**CNQX**), the non-NMDAR competitive antagonist, and (2R)-amino-5-phosphonovaleric acid (APV) the NMDAR antagonist. This prolonged activity is consistent with extrasynaptic actions mediated by spillover, and it is conceivable that a similar phenomenon occurs at $GABA_A$Rs on low-threshold primary afferents, thus explaining the long-lasting nature of PAD. In cerebellar granule cells, tonic extrasynaptic $GABA_A$ receptor signaling is found and related to GABA trapped within cerebellar glomeruli (Brickley, Cull-Candy et al. 1996; Wall and Usowicz 1997). Similar glomeruli have been widely identified in spinal cord associated with primary afferents (Alvarez 1998).

Figure 5 Putative Mechanisms for 'Direct' PAD



Potential mechanisms for PAD that are not mediated by the traditionally assumed trisynaptic pathway include disynaptic pathways mediated by last order GABAergic and/or cholinergic interneurons; non-spiking dendroaxonic synaptic effects onto primary afferent terminals; and production of PAD from one afferent population directly onto another.

One major concern immediately arises. If PAD is mediated by direct activation of $GABA_A$ receptors, then GABA must be co-released from glutamatergic primary afferent terminals, such that postsynaptic effects are glutamatergic and presynaptic effects are GABAergic. While this may seem heretical, there is clear evidence at other CNS sites of traditional glutamatergic synapses co-releasing GABA (Gutierrez, Romo-Parra et al. 2003), and of traditional GABA/glycine synapses co-releasing glutamate (Gillespie, Kim et al. 2005). Nonetheless, GABA/glutamate co-release from primary afferents remains doubtful, as labeling studies have not found evidence for GABA in primary afferents (Todd and McKenzie 1989; Todd and Spike 1993; Todd, Watt et al. 1996).

1.1.2.6 Aims and Objectives

This thesis investigates the ability of afferents to produce DRPs through more direct mechanisms than the typically assumed trisynaptic pathway. Chapter 2 outlines the difficulty in interpretation of pharmacological profiling of receptors in the Cys-loop family of ligand gated ionotropic receptors. The results presented suggest that a portion of the DRP, once thought to be solely $GABA_A$ergic, may in fact be nicotinic in nature. Additionally, Chapter 2 examines the PAD following block of ionotropic glutamate antagonists, which would presumably block transmission from primary afferents. I show that PAD may be more directly mediated by afferent release of non-glutamate transmitters leading to PAD of other afferents. Chapter 3continues to examine more direct forms of PAD; I demonstrate that much of the afferent-evoked PAD remains after pharmacological block of trisynaptic pathways, but that the remaining DRP is still blocked by $GABA_A$ antagonists.

In addition to showing that DRPs can be produced by mechanisms other than a trisynaptic pathway, in Chapter 4 I describe that a large component of the DRP is produced independent of $GABA_A$Rs and, in fact, independent of GABA. Block of a significant portion of the DRP after application of several nAChR antagonists supports the hypothesis that cholinergic receptor

activation is involved in the evoked DRP, and that this is mediated by specific subtypes of nAChRs. In particular, α9 and α10 subunit-containing nAChRs – both present in primary afferents – are sensitive to the $GABA_AR$ antagonists, bicuculline and picrotoxin (Lips, Pfeil et al. 2002; Alexander, Mathie et al. 2007), which have traditionally been used to demonstrate that PAD is a result of $GABA_A$ receptor activation. Some primary afferents (Tooyama and Kimura 2000; Bellier and Kimura 2007; Yasuhara, Aimi et al. 2008) and dorsal horn interneurons (Phelps, Barber et al. 1984) are cholinergic and may produce a DRP by activation of nAChRs. I also show that nAChRs are found directly on primary afferent terminals, by blocking all synaptic transmission and spiking (in the presence of the voltage-gated sodium channel blocker, tetrodotoxin) and showing that ACh application directly depolarizes afferent terminals. This depolarization can be blocked by subunit-specific nAChR antagonists. I also examine nicotinic AChR contribution to primary afferent stimulus evoked effects onto specific afferent classes. Combined, these studies elucidate previously unidentified cholinergic mechanisms producing PSI of primary afferents.

1.2 General Methodology

All procedures were approved by the Emory University Institutional Animal Care and Use Committee. Midsagittally-hemisected spinal cords were isolated from Sprague–Dawley rats aged from postnatal days 6-12 and were prepared for *in vitro* experiments as described previously (Machacek and Hochman, 2006;Shay et al., 2005) or from mice of the same age from the either the FVB or balb/c background. Recordings were made in oxygenated artificial cerebrospinal fluid (**aCSF**) containing (in mM): NaCl 128; KCl 1.9; D-glucose 10; $MgSO_4$1.3; $CaCl_2$ 2.4; KH_2PO_4 1.2; and $NaHCO_3$ 26. The *in vitro* preparation allows for exquisite solute and neurochemical control of afferent/spinal cord systems for detailed mechanistic studies on their function.

1.2.1 Stimulation of Afferents

Dorsal lumbar roots (L3, L4 or L5), peripheral muscle (e.g. posterior biceps, semitendonosus) and/or cutaneous nerves (e.g. sural, superficial peroneal) are left intact for stimulation of afferent fibers broadly separated by electrical stimulus intensity.

For the dorsal root stimulation experiments, afferent volleys are recorded antidromically distal to the site of stimulation to identify and monitor fiber populations recruited (Shay, Sawchuk et al. 2005). For peripheral nerve stimulation, the fastest afferent volleys are recorded in the sciatic nerve, a dorsal root that has been cut away from the spinal cord, or in an electrode near the dorsal root entry zone before the onset of the DRP. Stimuli are then expressed in multiples of the value that just elicits an afferent volley (threshold; **T**) and are delivered at intensities four times threshold and 10T, to examine low versus high threshold afferent-evoked actions. In addition, constant current stimuli at 100μA, 100μs will be delivered for supramaximal recruitment of both low and high threshold afferents. Both methods are employed by different investigators of

sensory and motor function, and it is important for initial experiments to convey the experimental results in relation to both backgrounds.

1.2.2 Recordings

DRPs were recorded from cut dorsal root filaments or in intact roots via *en passant* nerve suction with suction electrodes. Ventral root potentials (**VRPs**) and accompanying reflexes were recorded to monitor motor output. We used 2M Na^+-citrate sharp electrodes to record intraspinal extracellular field potentials (EFPs). Sites of peak EFP amplitudes represent dominant termination sites of primary afferents. This electrode was then used for intraspinal microstimulation to study effects of drugs on the amplitude of back-propagating spikes in dorsal roots or peripheral nerves, and on the microstimulation evoked DRP, as direct measures of changes in fiber excitability.

Raw data were collected through a differential amplifier, digitized at 5-15 kHz (Digidata 1322A 16-Bit DAQ, Axon Instruments), and recorded with pClamp software (v. 10.0, Axon Instruments, Molecular Devices, U.S.A.) and stored in a PC for off-line analysis with pClamp software or a custom MATLAB program.

More detailed descriptions of the experimental methodology precede each chapter to encompass the experimental design employed to collect the data described in each respective therein.

Chapter 2 Presynaptic inhibition of primary afferents by depolarization: Observations supporting non-traditional mechanisms

(Adapted from (Hochman, Shreckengost et al. 2010))

2.1 Abstract

Primary afferent neurotransmission is the fundamental first step in the central processing of sensory stimuli and is controlled by pre- and post-synaptic inhibitory mechanisms. Presynaptic inhibition (PSI) is probably the more powerful form of inhibitory control in all primary afferent fibers. A major mechanism producing afferent presynaptic inhibition is via a channel-mediated depolarization of their intraspinal terminals which can be recorded extracellularly as a dorsal root potential (DRP). Based on measures of DRP latency it has been inferred that this primary afferent depolarization (PAD) of low-threshold afferents is mediated by minimally trisynaptic pathways with pharmacologically-identified GABAergic interneurons forming last-order axo-axonic synapses onto afferent terminals. There is still no 'squeaky clean' evidence of this organization. This chapter describes recent and historical work that supports the existence of PAD occurring by more direct pathways and with a complex pharmacology that questions the proprietary role of GABA and $GABA_A$ receptors in this process. Cholinergic transmission in particular may contribute significantly to PAD, including via direct release from primary afferents.

2.2 Introduction

2.2.1 Presynaptic inhibition (PSI) of primary afferents

The spinal cord is the neural interface between body and brain, receiving a continuous barrage of sensory information via primary afferents that requires central mechanisms to limit and channel their signaling. The intraspinal terminals of primary afferents, located predominantly in the dorsal horn (laminae I-VI) represent the first CNS site for this control, and here it appears to be

regulated by frighteningly complex processes (Rudomin and Schmidt 1999). A principal mechanism for reduction of afferent neurotransmission is via prior depolarization of their terminals, termed primary afferent depolarization (**PAD**) which paradoxically reduces transmitter release. GABAergic inhibitory interneurons are thought to mediate PAD via a trisynaptic circuit (Figure 6A). Using pumps, sensory neurons maintain the chloride gradient to be depolarizing so that activation of intraspinal $GABA_A$ receptors results in the observed PAD (Figure 3). PAD can be measured experimentally following its antidromic electrotonic spread to dorsal roots as a dorsal root potential (**DRP**).

Sensory afferents arising from skin or muscle can be broadly separated into two categories: low-threshold and high-threshold. Large-diameter/fast-conducting myelinated fibers require lower electrical stimulus intensities (**low-threshold**) for recruitment than smaller-diameter un- or thinly myelinated fibers (**high-threshold**). Low-threshold afferents are non-pain encoding cutaneous Aβ and muscle group Ia, Ib & II mechanoceptors, and the presynaptic inhibitory control of these fibers is the focus of this chapter.

As alluded to above, the classical electrophysiological studies obtained indirect experimental evidence that afferent-evoked presynaptic inhibition of low-threshold afferents is produced via a minimally tri-synaptic pathway involving last order GABAergic inhibitory interneurons. This chapter will underscore limitations in this model of afferent-evoked PAD, and then provide evidence to suggest that: (i) more direct feedback mechanisms are found, (ii) that GABA may not be the only transmitter involved, (iii) and that the classical $GABA_A$ receptor may also not be the only receptor involved. If true, the results further expand the mechanisms by which somatosensory information processing is controlled.

2.2.2 Some limitations with the current model of afferent-evoked PAD

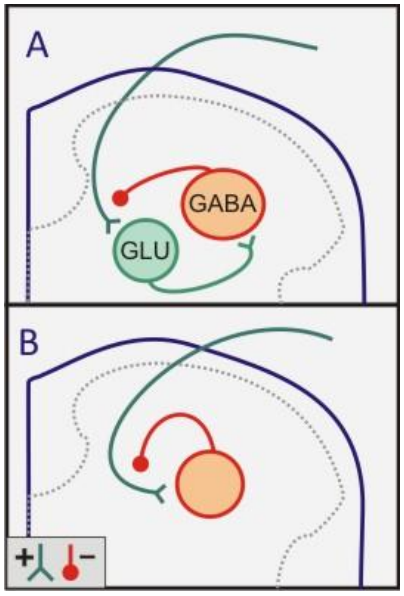
Although it is assumed that anatomically-identified GABAergic axo-axonic synapses onto

identified afferents arise from the GABAergic interneurons mediating afferent activity-evoked PAD, direct electrophysiological evidence is still lacking (Eccles, Schmidt et al. 1963; Barker and Nicoll 1973; Rudomin and Schmidt 1999). Putative PAD interneurons have historically been identified based on their extracellular spike being temporally coincident with the DRP using the technique of spike-triggered averaging (Rudomin, Solodkin et al. 1987; Jankowska and Riddell 1995). However, as recently stated by Rudomin (2009) on their earlier work; "One of the problems with the interpretation of these findings was that the spontaneous interneuronal activity of the interneurons assumed to mediate PAD of muscle afferents appeared in synchrony with a negative CDP [cord dorsum potential] which started 25–50 ms before the interneuronal activity used to trigger the DRP and VRP recordings (Rudomin 2009)." We concur with Wall who argued in 1998 that "we should remain cautious in the identification of those nerve cells which lead singly or in a chain to the generation of PAD (Wall 1998)."

If PAD occurred via a trisynaptic pathway, it is worth questioning why PAD is not reduced by anesthetics like pentobarbital, which decrease excitatory synaptic activity in all neurons (Franks and Lieb 1994). Several of the studies of PAD by Rudomin, Eccles, Jankowska and their colleagues used pentobarbital as the anesthetic (Eccles 1964; Jankowska, McCrea et al. 1981; Jimenez, Rudomin et al. 1984). As stated by Eccles et al (Eccles, Schmidt et al. 1963); "*The cutaneous volleys produce DRPs at such deep anaesthesia that their occurrence in the absence of all interneuronal activity has been suggested (Wall, 1958)* (Wall 1958). *If this were established it would falsify the hypothesis that presynaptic inhibition is due to … activation of specific interneuronal pathways.*" While it can be countered that barbiturates would also strengthen PAD by directly potentiating $GABA_A$ receptor activity (Franks and Lieb 1994), in order for afferents to evoke PAD tri-synaptically, they must go through interneurons, and we now know that pentobarbital potently inhibits glutamatergic transmission (AMPA receptor IC_{50} ~50μM ≈ 1mg/kg) (Marszalec and Narahashi 1993; Jackson, Joo et al. 2003).

If the classical minimally trisynaptic interneuronal circuit does not contribute much to the observed PAD, how else might PAD be produced? The simplest answer is disynaptically. This would explain the circuit in the dorsal horn, where the location of all putative interposed interneurons is always where primary afferents terminate and never elsewhere, suggesting a very spatially restricted circuit, where the postsynaptic cells may likely also be the cells leading to PAD of primary afferents(Jankowska, McCrea et al. 1981; Jankowska and Riddell 1995)(see (Hughes, Mackie et al. 2005) for ventral horn). Disynaptic circuits mediating PAD of low-threshold afferents appear highly-likely. Lamina III cholinergic interneurons receive input from myelinated and unmyelinated cutaneous afferents and appear to be interposed in negative feedback disynaptic circuits back onto the same primary afferents (Olave, Puri et al. 2002). Moreover recent intracellular recordings from two intermediate zone interneurons monosynaptically-excited by group I and II muscle afferents were intracellularly labeled and shown to project axons directly apposed to primary afferent terminals (i.e. disynaptic circuit) (Bannatyne, Liu et al. 2009). "These two interneurons are therefore likely to be the first two examples of PAD interneurons in higher vertebrates to be characterized electrophysiologically as well as labeled intracellularly" (Bannatyne, Liu et al. 2009) although it was not shown that they were directly linked to PAD. Importantly, based on latency measures this circuit was considered trisynaptic (Figure 6) (Eccles, Magni et al. 1962; Jankowska, McCrea et al. 1981).

Figure 6 Trisynaptic versus Direct PAD



A. Classical minimally trisynaptic network for presynaptic inhibition of low-threshold muscle & cutaneous afferents. B. Recent evidence also supports more direct disynaptic pathways

Another possibility for PAD generation is disynaptically via a non-spiking dendroaxonic microcircuitry, which has been suggested but not demonstrated by Hounsgaard based on the observation of a TTX-insensitive PAD evoked by stimulation of high threshold afferents in the turtle (Russo, Delgado-Lezama et al. 2000). Alternatively, the actions could be direct via homosynaptic or heterosynaptic negative feedback of transmitter released from primary afferents including via spillover mechanisms (Kullmann, Ruiz et al. 2005) (Figure 5). Presynaptic autoreceptors represent a dominant form of PSI in CNS neuron terminals (Engelman and MacDermott 2004), and this much simpler mechanism may explain the presence of PAD onto their own and related terminals (Rudomin and Schmidt 1999) as well as why PAD is comparatively long-lasting. However, direct feedback would be inconsistent with a GABAergic mechanism since primary afferents would need to release GABA and labeling studies have failed to support GABA as a transmitter localized in primary afferents (Todd and Spike 1993). Another transmitter would need to be released from primary afferents and be capable of activation of a $GABA_A$-like receptor for such direct actions. Possibilities include acetylcholine, taurine and β-alanine as elaborated below in Section 2.4.

2.3 Further peculiarities of PAD

2.3.1 Is GABA really the transmitter producing afferent-evoked PAD?

The existence of GABAergic synapses onto primary afferents clearly demonstrates that at least some GABAergic projections must exist that produce presynaptic inhibition of primary afferents (Alvarez 1998; Hughes, Mackie et al. 2005). However, they may not be associated with <u>all</u> forms of evoked PAD. One possible organization is that GABAergic interneurons are predominantly responsible for the PAD generated by descending systems but not by several afferent systems. Early work from two groups demonstrated that the evoked efflux of neuronal [3H]-GABA in amphibian spinal cord arose from Ca^{2+}-dependent release following stimulation of descending

spinal tracts but not following stimulation of primary afferents (Roberts and Mitchell 1972; Collins 1974). The marginal release of [3H]-glutamate following primary afferent stimulation suggests that afferent activation may simply have been insufficient to evoke GABA release (Roberts and Mitchell 1972). The evidence for GABA as the transmitter responsible for afferent evoked PAD is: (i) the existence of GABAergic axo-axonic synapses on primary afferents (Alvarez 1998), (ii) afferent-evoked PAD is blocked by $GABA_A$ receptor antagonists (Nicoll and Alger 1979; Rudomin and Schmidt 1999), and (iii) drugs blocking GABA synthesis and degradation depress or facilitate afferent-evoked PAD respectively(Levy 1977; Nicoll and Alger 1979). This latter point is highly suspect. The hydrazines used as blockers of synthesis are very non-specific, and the inhibitors of GABA degradation are also non-specific and demonstrated a facilitatory effect in only one of 4 studies (see references in (Nicoll and Alger 1979)). Potential weaknesses in the evidence of the first two more critical points are described below.

2.3.2 Is the $GABA_A$ receptor really the receptor activated to produce afferent-evoked PAD?

Regarding GABAergic terminals onto primary afferents, "little can be said to link the presynaptic terminals visualized with electron microscopy with particular spinal cord neurons or axonal arborizations" (Alvarez 1998). Additionally, while a high proportion of terminals onto primary afferents are immunopositive for GABA or its synthesis enzyme glutamic acid decarboxylase (**GAD**) this does not necessarily equate to a presence of postsynaptic $GABA_A$ receptors. For example, no changes were observed in autoradiographic mapping of spinal $GABA_A$ receptor ligands after degeneration of dorsal root fibers (Castro-Lopes, Malcangio et al. 1995) with no evidence of α2/α3 labeling in primary afferent terminals using both EM and light microscopy (Alvarez 1998). Based on the above, it is instead possible that postsynaptic receptors to $GABA^+$ presynaptic terminals contain bicuculline/picrotoxin-sensitive glycine (α1, α2, β), nicotinic (α9, α10), $GABA_A$-rho, or $5HT_{3A}$, receptor subunits. $GABA_A$, glycine, ACh nicotinic and 5-HT_3 receptors all comprise the Cys-loop family of transmitter-gated ion channels with common

molecular architecture (Connolly and Wafford 2004), so overlapping pharmacological actions are unsurprising (see below), and that PAD may be mediated by a combination of these transmitters and receptors.

2.3.3 Could another Cys-loop family receptor produce PAD?

The pharmacology of low-threshold PAD has been studied in detail (Eccles, Schmidt et al. 1963; Levy 1977; Nicoll and Alger 1979), but not for a long time. Past interpretations of results were based on the assumption that the drugs used had rather specific actions. As described below there are nicotinic, $5HT_3$ and glycine receptor subunits with sensitivity to traditional $GABA_A$ antagonists.

2.3.3.1 Nicotinic receptors (nAChRs)

The nAChR is a non-selective cation channel. Primary afferents contain numerous nAChR subtypes (Genzen, Van Cleve et al. 2001; Lips, Pfeil et al. 2002). Bicuculline (IC_{50} = 0.8μM) and strychnine (IC_{50} = 0.02μM) potently inhibit the α9 nicotinic ACh receptor subunits (Rothlin, Katz et al. 1999) and nAChR α10 subunits, which co-assemble with α9 to form a functional receptor, also have sensitivity to strychnine and bicuculline. Picrotoxin is also able to inhibit α3-containing and α7 nAChRs with IC_{50} values of 96.1 +/- 5.5 and 194.9 +/- 19.2 μM for the α3β4 and α7, respectively (Erkkila, Weiss et al. 2004). This raises the possibility that alongside $GABA_A$ergic mechanisms, a part of PAD is nicotinic receptor-mediated. However, PAD is barely affected by strychnine in the *in vitro* preparation described below (Figure9), and does not appear to affect PAD in the cat (Jimenez, Rudomin et al. 1987). Critically, only larger diameter (i.e. low threshold) DRG neurons contain α-bungarotoxin binding sites (examined in rat, cat, monkey, and human) (Ninkovic and Hunt 1983). These neurons represent at least 12% of large diameter afferents, and appear to selectively project to lamina III, which is the projection site of Aβ cutaneous afferents (Willis and Coggeshall 1991) and the presumed location of interneurons

interposed in the PAD pathway of cutaneous afferents (Jankowska, McCrea et al. 1981). Dorsal horn cholinergic interneurons (Barber, Phelps et al. 1984) are also located there, making them well positioned to produce PAD (Todd 1991; Alvarez 1998).

2.3.3.2 5HT3A receptors

The serotonin $5HT_3$ receptor is a non-selective cation channel. Bicuculline (IC_{50} 20 μM) and picrotoxin (IC_{50} 30 μM) antagonize $5HT_{3A}$ receptors at pharmacologically relevant doses (Das, Bell-Horner et al. 2003). $5HT_{3A}$ receptors are strongly expressed in myelinated primary afferents (Zeitz, Guy et al. 2002).

2.3.3.3 Glycine receptors

The inhibitory glycine receptor is principally permeable to chloride. In retinal ganglion cells, glycine receptors are potently blocked by traditional $GABA_A$ receptor antagonists (bicuculline, gabazine and picrotoxin) as were their α1 and α2 glycine receptor subunits expressed in HEK cells (Wang and Slaughter 2005) with α2 subunits showing relative strychnine insensitivity (Han, Li et al. 2004). Beta subunits co-assembled with α subunits lead to greater bicuculline and gabazine sensitivity but reduced picrotoxin sensitivity (Li and Slaughter 2007).

2.3.3.4 Unusual receptor subunit assemblies

There is also evidence of promiscuous subunit assembly between different members of the Cys-loop family. For example, $GABA_A$ γ2s can co-assemble with glycine α subunits to form functional glycine receptors (Li and Slaughter 2007). There is also evidence of co-localization of α1 glycine receptor subunit with the $GABA_A$ γ2 and $GABA_A$ρ receptor subunits in spinal cord but not retina (Frazao, Nogueira et al. 2007). Also, glycine α and $GABA_A$ γ subunit mRNA may be co-expressed in large DRG neurons (Furuyama, Sato et al. 1992).

In sum, earlier conclusions that classical $GABA_A$ receptors are essential for PAD based on pharmacological observations can no longer be viewed as definitive.

2.4. Could another transmitter be released to produce PAD?

2.4.1 Acetylcholine

Lamina III is a projection site of Aβ and Aδ cutaneous afferents (Willis and Coggeshall 1991) and is the likely location of interneurons interposed in PAD of cutaneous afferents (Jankowska, McCrea et al. 1981).Intriguingly, it is also the predominant location of dorsal horn cholinergic interneurons (Olave, Puri et al. 2002) - well positioned to contribute to PAD (Todd 1991; Alvarez 1998). Acetylcholine (**ACh**) and GABA co-exist in a population of dorsal horn interneurons (Todd 1991) and are found in ~25% of the synapses presynaptic to afferent axons (Alvarez 1998). Thus, there is ample anatomic evidence to support ACh as a transmitter contributing to PAD.

Intriguingly, while the early literature did not identify acetylcholine (**ACh**) as a neurotransmitter in primary afferents (Willis and Coggeshall 1991), recent work strongly challenges these studies. First, an alternative splice variant of the ACh synthesis enzyme choline acetyltransferase (**ChAT**) has been identified. Peripheral ChAT (**pChAT**) is preferentially localized in peripheral neurons including in dorsal root ganglia (**DRG**) (Tooyama and Kimura 2000), and has sufficient enzyme activity in the adult rat DRG to produce physiological concentrations of Ach (Bellier and Kimura 2007). PChAT is expressed in both small and large diameter primary afferents (Bellier and Kimura 2007) and immunolabeling studies identify pChAT in myelinated primary afferents that project via dorsal columns to brainstem dorsal column nuclei (Yasuhara, Aimi et al. 2008). DRG neurons have also been shown to express the vesicular ACh transporter (**VAChT**) preferentially in large-diameter DRG neurons (Tata, De Stefano et al. 2004) as well as the ACh degradative enzyme acetylcholinesterase (**AChE**) (Willis and Coggeshall 1991; Bellier and Kimura 2007). Widespread ChAT labeling in DRG was also observed in mice, including ChAT-GFP BAC transgenic mice (Tallini, Shui et al. 2006), and treatment with antisense oligonucleotides reduced labeling (Matsumoto, Xie et al. 2007).

2.4.2 Taurine and β-alanine

Taurine is released from neurons and glia upon hypo-osmotic swelling and is believed to behave chiefly as an osmoregulator. However, in spinal cord, microdialysis experiments reveal that taurine and β-alanine are released following sciatic nerve stimulation in concentrations comparable to glutamate (Paleckova, Palecek et al. 1992). Taurine and β-alanine are weak agonists at GAB_AA receptors (Nicoll and Alger 1979), though taurine may also be a potent activator of extrasynaptic GAB_AA receptors (Jia, Yue et al. 2008). Taurine and β-alanine also directly depolarize primary afferents in the in vitro rat spinal cord (Evans 1978). As in frog, responses are blocked by picrotoxin (50 μM) and bicuculline (5 μM). Like GABA and glycine, taurine and β-alanine are transported via Na+-dependent high affinity uptake systems with significant sequence homology to the GABA and glycine transporters systems (Smith, Borden et al. 1992; Liu, Lopez-Corcuera et al. 1993). More recently, taurine labeling has been demonstrated in spinal cord with greatest density in superficial dorsal horn, but also associated with myelinated axon terminals (Lee, Renno et al. 1992). Additionally, cysteine dioxygenase and sulfinoalanine decarboxylase, two critical enzymes in the taurine synthesis, are expressed in primary afferents (Ledoux, Xu et al. 2006) and synaptic vesicles contained within synaptosomes are enriched in taurine (Bonhaus, Lippincott et al. 1984).

In sum, primary afferents may co-release substances that act on receptors having $GABA_A$-like pharmacology.

2.5 Experimental support for the existence of non-traditional mechanisms serving afferent-evoked PAD

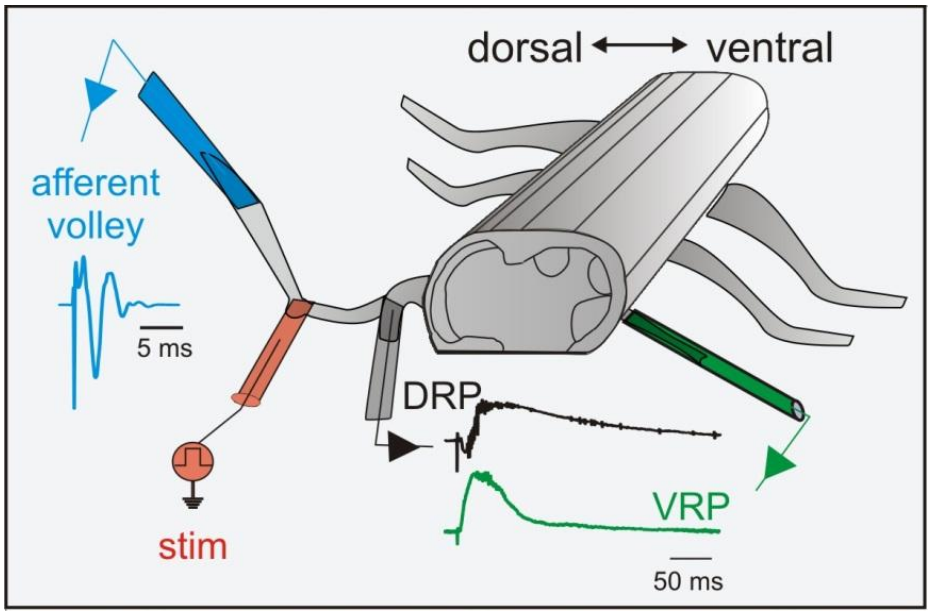
2.5.1 The hemisected spinal cord maintained in vitro.

Jorge Quevedo has developed a preparation to show that afferent-evoked PAD in the isolated *in vitro* spinal cord of young mouse and rat evoked by stimulation of intact peripheral nerves has

many characteristics similar to those observed in the adult cat (Garcia and Quevedo 2001). The *in vitro* model has afforded us the ability to examine the mechanisms serving PAD with greater pharmacological precision. For example, in vivo studies on PAD mechanisms undertaken in the presence of anesthetics (e.g. α-chloralose and barbiturates) are expected to alter the mechanisms generating PAD (Franks and Lieb 1994; Harrison and Krasowski 2000). Moreover, *in vivo* studies are often necessarily undertaken in the presence of paralytic agents like gallamine which act on nicotinic receptors and also alter the DRP (Grinnell 1970).

The results from experiments herein described were undertaken both in mouse and rat (P6-15). The results obtained were the same in both species but only experiments in rat are shown. The experimental setup is shown in Figure 7. The L5 dorsal root is stimulated, and DRPs are recorded from the same (homonymous) or adjacent dorsal root (L4; heteronymous). For ease of viewing only one DRP is shown in the figures displayed. All actions were studied from putative low threshold afferents, typically at 4 times the stimulus intensity that just elicits an afferent volley (threshold; 4T) or at 100 μA, 100 μs. The afferent volley was monitored to observe recruitment of low-threshold afferents and to ensure afferent volley amplitude is unaffected by drug applications so that observed actions are due to events in the spinal cord. Also, whenever tested all recruited afferents and the DRP were completely blocked with low dose TTX (100 nM) excluding a contribution from high-threshold TTX insensitive afferents (Russo, Delgado-Lezama et al. 2000).

Figure 7 Methods for studying PAD-related mechanisms



Midsagittally-hemisected spinal cords were isolated from mice or rats aged from postnatal days 7–14 and were prepared for in vitro experiments as described previously (Shay, Sawchuk et al. 2005). Stimulation is via the L5 dorsal root at 100μA, 100μs or at 4T. A single stimulus produces a DRP that is almost entirely blocked by bicuculline. Low threshold activation is confirmed by measuring the afferent volley produced by this stimulation and its TTX sensitivity.

2.5.2 Low-threshold DRPs require $GABA_A$-like receptor activation.

PAD generated by low-threshold afferent stimulation is blocked by $GABA_A$ receptor antagonists, as shown previously in cat (Jimenez, Rudomin et al. 1987) rat (Evans 1978; Vinay, Brocard et al. 1999) and frog (Nicoll and Alger 1979). Here, we also demonstrate that the DRP in this preparation is blocked with bicuculline (Figure 8A).

2.5.3 Bicuculline-sensitive DRPs remain after greatly restricting synaptic transmission.

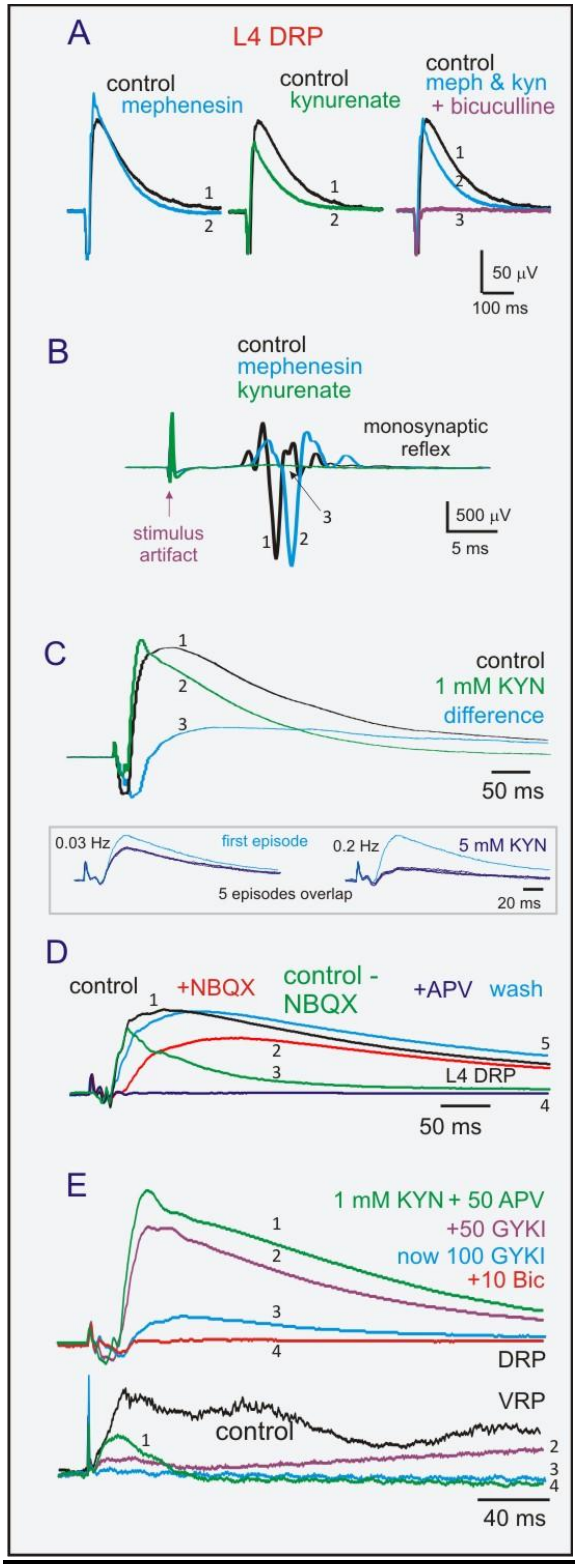
It is widely believed that PAD of low-threshold afferents is generated by minimally trisynaptic pathways (Rudomin and Schmidt 1999). We explored whether PAD can be generated via more direct actions of primary afferent transmitter release using mephenesin. Several labs have used mephenesin (Lev-Tov and Pinco 1992; Quinlan and Kiehn 2007) to isolate monosynaptic components. We observed that low threshold DRPs remain after curtailing di- and poly-synaptic transmission with 1 mM mephenesin (Figure 8A). This is consistent with an earlier *in vivo* report on the actions of mephenesin (Farkas, Tarnawa et al. 1989), never cited in reviews on PAD. Concomitant recordings from the ventral root showed that the monosynaptic reflex amplitude was unaffected (Figure 8B). Thus, trisynaptic and probably even disynaptic pathways were not required to generate the early component of the DRP. Overall these results demonstrate that PAD of low-threshold primary afferents can occur by more direct synaptic mechanisms, including the possibility of direct negative-feedback or non-spiking dendroaxonic pathways (Figure 5).

2.5.4 DRPs can remain even after the block of glutamatergic transmission.

Contrary to the dogma that primary afferent transmission is glutamatergic, much of the DRP remains after blockade of excitatory synaptic transmission with the ionotropic glutamate receptor antagonist kynurenate at a dose thought to fully block ionotropic glutamate receptors (Jahr and Yoshioka 1986) (Figure 8C). This is consistent with an earlier report in the isolated sacrococcygeal cord of adult rat (Evans and Long 1989). To confirm kynurenate block of

glutamatergic transmission, we also showed that kynurenate always completely blocked the monosynaptic reflex (Figure 8B) and almost blocked all of the sub threshold ventral root potential (**VRP**; Figure 8E). On several occasions, kynurenate DRP block could be more substantial (e.g. at 5 mM). This is at least also partly explained by a sensitivity of the kynurenate-resistant DRP to stimulus frequency (we stimulated at a range between 0.033-0.2 Hz) (Figure 5C inset), but may also relate to recruitment of different afferents depending on the axons selected with the suction electrode. We also tested the actions of the non-NMDA receptor antagonists NBQX and GYKI52466. In the presence of the NMDA receptor antagonist APV, NBQX (Figure 8D) but not GYKI (Figure 8E) completely blocked the DRP. Note GYKI almost completely blocked the VRP at 50 μM while the DRP was only partially affected (Figure 8E, bottom). The differential sensitivity of glutamate receptors antagonists to afferent-evoked DRPs vs. reflex actions has been observed previously in vivo (Farkas and Ono 1995). Kynurenate insensitive afferent-evoked synaptic responses have also been reported in some dorsal horn neurons (Schneider and Perl 1988). These variable results with the glutamate receptor antagonists suggest that a component of PAD may not require glutamatergic synaptic transmission. Moreover, some glutamate receptor antagonists could instead have direct actions at $GABA_A$-like receptors. For example, the quinoxalines (CNQX, DNQX and NBQX) can act as antagonists of homooligomeric α1 and α2 glycine receptor subunits (NBQX IC_{50} 5 μM > DNQX > CNQX) (Meier and Schmieden 2003) and CNQX has a significant non-competitive blocking effect on the $GABA_A$ receptor channel complex between 20-50 μM (Jarolimek and Misgeld 1991).

Figure 8 Glutamate antagonists differentially affect the DRP

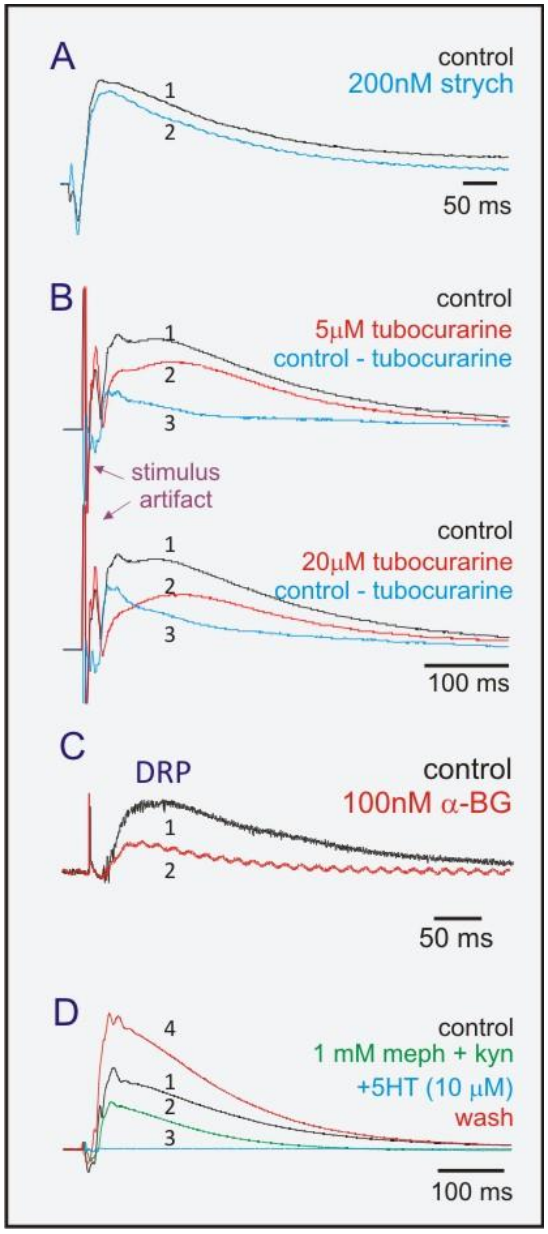


A, B. Low threshold DRPs remain after block both of polysynaptic transmission and ionotropic glutamate receptors. A. While mephenesin (1 mM) only slightly modifies the DRP, kynurenate (1 mM) reduced amplitude and more preferentially the duration of the DRP. Subsequent addition of bicuculline (10 μM) fully blocked the DRP. B. Note that mephenesin (1 mM) slowed onset of monosynaptic reflex but did not alter reflex amplitude. Kynurenate (1 mM), the broad spectrum glutamate ionotropic receptor antagonist completely blocked the reflex (green trace). C-E. Differential sensitivity of DRPs to glutamate receptor antagonists. C. Kynurenate blocks the longer latency component of the DRP (difference trace). Additionally, kynurenate actions are stimulus frequency-sensitive. The DRP remaining after 5 mM kynurenate is greatly depressed by an afferent stimulation frequency of 0.2 Hz (inset). D. The selective AMPA receptor antagonist NBQX (10 μM) blocks the short latency component (see control - NBQX trace). Subsequent application of APV (50 μM) reversibly blocks the DRP. E. APV has no effect subsequent to kynurenate application showing that kynurenate adequately blocked NMDA receptors. Like NBQX, GYKI52466 is a selective AMPA receptor antagonist but cannot fully block the DRP (after kynurenate and APV have already been added) even at a dose of 100 μM. Note the VRP is nearly completely blocked by 50 μM GYKI demonstrating a differential sensitivity of GYKI to VRP vs. DRP. Numbers adjacent to traces reflect order of application.

2.5.5. Typical and atypical pharmacology of the DRP.

We explored the sensitivity of the DRP to multiple ligands. Low doses of the glycine receptor antagonist strychnine (200 nM) partly blocked the DRP (Figure 9A). Surprisingly, the nicotinic receptor antagonist tubocurarine selectively blocked the early component of the DRP in a dose-dependent manner (Figure 9B). Moreover, the highly-selective nicotinic receptor antagonist α-bungarotoxin also depressed the DRP (Figure 9C). Sensitivity of the DRP to tubocurarine and α-bungarotoxin is consistent with actions on α9α10 nicotinic receptor subunits (Rothlin, Katz et al. 1999; Alexander, Mathie et al. 2007). Remarkably, after pharmacologic isolation of the DRP with mephenesin and kynurenate, 5HT could also completely and reversibly block the DRP (Figure 9D). While 5-HT may be directly depressing the afferents generating the DRP, it may also be acting directly by blocking α9α10 nicotinic receptors (Rothlin, Lioudyno et al. 2003). Overall these results suggest that the receptor(s) responsible for PAD have a hitherto unrecognized complex pharmacology. The common thread of these antagonists is their demonstrated actions on the Cys-loop family of transmitter-gated ion channels with common molecular architecture ($GABA_A$, glycine, nicotinic and $5\text{-}HT_3$) (Alexander, Mathie et al. 2007).

Figure 9 Sensitivity of Putative $GABA_A$-like receptor to various ligands.



A. Strychnine blocks part of the early DRP. B. Tubocurarine blocks the DRP in a dose-dependent manner. Subtraction from control demonstrates that the early DRP is preferentially inhibited. C. α-bungarotoxin reduces the DRP. D. After synaptic isolation, 5HT reversibly blocks the DRP. 5-HT may be a competitive antagonist at the transmitter binding site that generates the DRP. Numbers adjacent to traces reflect order of application.

2.5.6. Many primary afferents are probably cholinergic.

If afferent-evoked PAD occurs in part by direct negative feedback, primary afferents must release transmitters that can directly activate $GABA_A$-like receptors. One possibility is ACh acting on '$GABA_A$-like' nicotinic receptors. As stated earlier, a growing body of evidence suggests that many primary afferents have a cholinergic phenotype (Tooyama and Kimura 2000; Matsuo, Bellier et al. 2005; Bellier and Kimura 2007; Yasuhara, Aimi et al. 2008). Work from our collaborator, Hirosi Kimura has shown that pChAT, AChE and the vesicular ACh transporter are immunodetected in DRG neurons (Figure 10A). Importantly, Kimura and colleagues have demonstrated that primary afferent axons that project via the dorsal columns have extensive pChAT labeling (Figure 10B). Moreover these afferents also project to deeper dorsal horn spinal laminae at sites consistent with termination of non-pain encoding low-threshold cutaneous afferents (Figure 10C).

2.5.7. Primary afferents express taurine and β-alanine.

Another possible mechanism for direct negative feedback of some afferents is the direct activation of $GABA_A$ receptors by $GABA_A$R agonists taurine or β-alanine released from primary afferents. While a single study inferred this for taurine (Lee, Renno et al. 1992), here we provide immunolabeling evidence for both amino acids. We have so far tested taurine in both rat and mouse and on both occasions numerous large DRG neurons were labeled (Figure 11A). Similarly, β-alanine was tested in mouse and labeling was also in large diameter cell bodies (Figure11B). Afferents with large diameter cell bodies equate to low threshold actions (Willis and Coggeshall 1991).

2.5.7.1 Taurine labeling is in a subpopulation of primary afferent glutamatergic synapses.

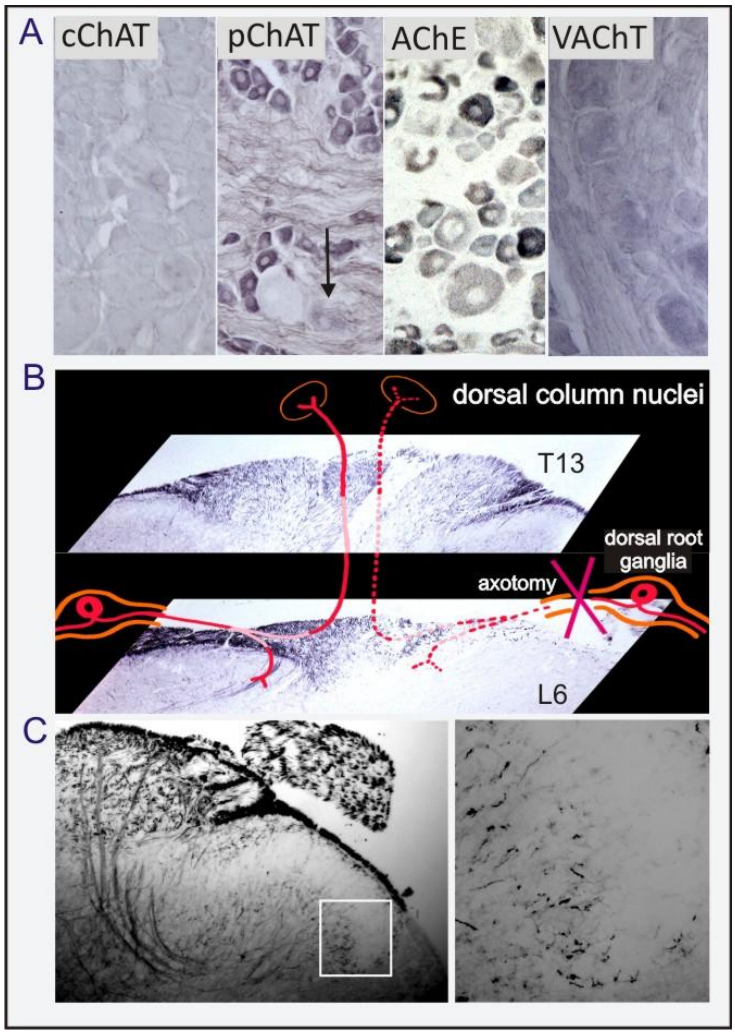
As observed previously (Lee, Renno et al. 1992) taurine labeling is dominant in the superficial dorsal horn and largely punctate (Figure 11C). Primary afferents are glutamatergic so co-labeling

for taurine and the vesicular glutamate transporter 1 (**vGluT1**) would support co-transmission (Alvarez, Villalba et al. 2004). Preliminary observations demonstrate that taurine punctate labeling partly co-localized to a subpopulation of VGluT1 primary afferent putative glutamatergic synapses in deeper dorsal horn (Figure 11D). These observations support the hypothesis that taurine is released from $vGluT1^+$ primary afferents via conventional synaptic mechanisms.

2.3. Conclusion

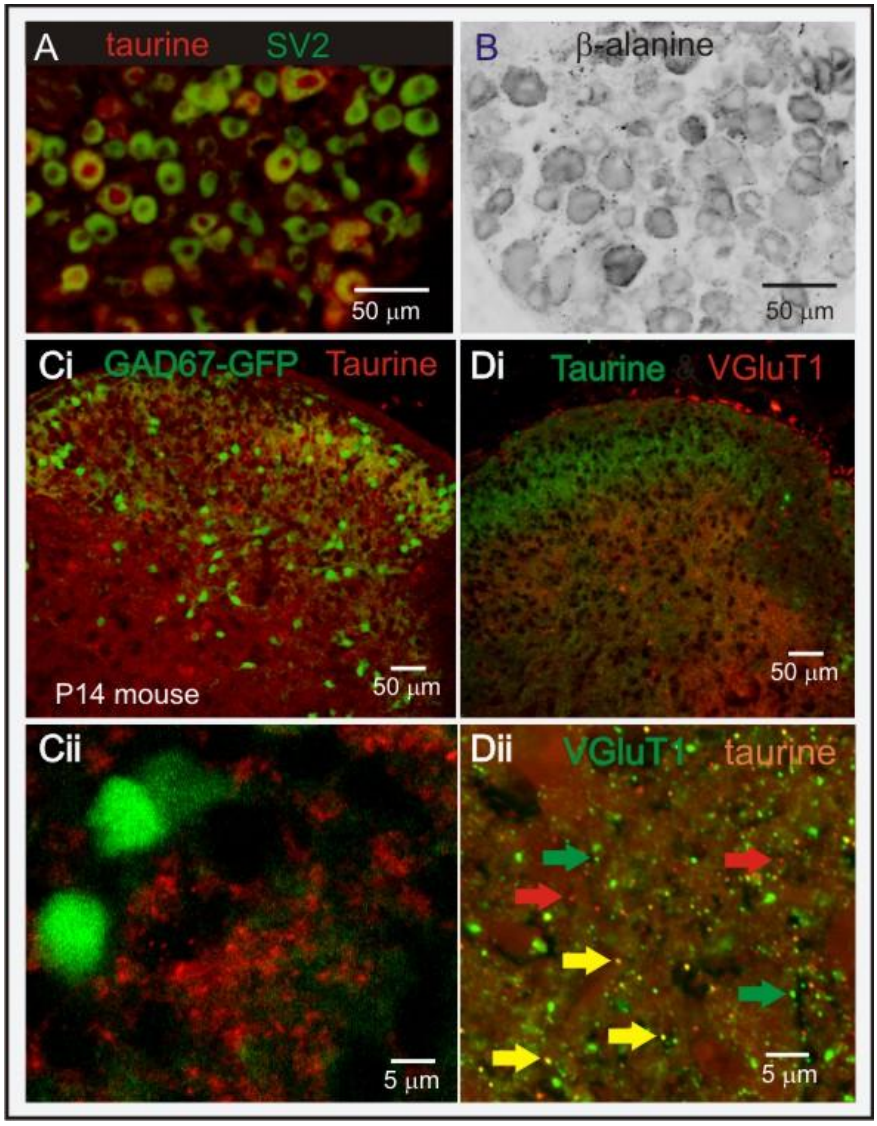
It is widely appreciated that presynaptic inhibition is "more powerful than postsynaptic inhibition in depressing the central excitatory actions of almost all primary afferent fibers" (Eccles 1964). Block of an impulse at the presynaptic terminal of a primary afferent would effectively arrest a signal that would require multiple inhibitory postsynaptic inputs to similarly depress. It has been assumed, though never directly demonstrated, that this primary afferent depolarization (**PAD**) of low-threshold afferents is mediated by a trisynaptic pathway and that GABAergic interneurons are essential (Rudomin and Schmidt 1999). We present experimental evidence in the context of previously published studies that PAD can also be generated by more direct synaptic pathways and may be at least partly independent of GABA and classical $GABA_A$ receptors. These findings suggest PAD mechanisms may be much more diverse than previously imagined, conceivably separated into distinct mechanisms for each genetically distinct afferent fiber population. If true, the current model of afferent-evoked PAD requires substantial updating.

Figure 10 Expression of pChAT, AChE and VAChT in DRG and pChAT in the spinal cord of adult rat.



A. Conventional antibody for ChAT (cChAT) does not label DRG neurons, but pChAT, AChE and the vesicular ACh transporter (weakly) are immunodetected. Weaker staining in some larger DRG neurons (arrow) is consistent with lower expression detected with RT-PCR (Bellier and Kimura 2007). B. pChAT immunolabeling in the dorsal columns. Note that after axotomy of the right L6 DRG, there is a complete loss of pChAT labeling in an associated band within the T13 dorsal column, verifying that all labeling arose from L6 primary afferents. C. $pChAT^{+}$ afferents project to deeper spinal laminae. Afferent entry and termination pattern is consistent with non-pain encoding cutaneous afferents.

Figure 11 Taurine/β-alanine labeling in primary afferents



A, B. Taurine/β-alanine labeling in DRG. A. Taurine labels a population of synaptic vesicle 2 (SV2)-labeled neurons. B. β-alanine labeling in DRG appears more widespread. C, D. Taurine has a distinctive labeling pattern in spinal cord. C. Taurine punctate labeling does not appear to overlap with $VGluT1^{+}$ synapses at low power. D. In another animal, higher power confocal imaging shows that taurine co-localizes with several $VGluT1^{+}$ putative primary afferent terminals in deep dorsal horn (laminae IV-V).

Chapter 3 Bicuculline-sensitive primary afferent depolarization remains after greatly restricting synaptic transmission in the mammalian spinal cord.

(Adapted from (Shreckengost, Calvo et al. 2010))

3.1 Abstract

Primary afferent neurotransmission is the fundamental first step in the central processing of sensory stimuli. A major mechanism producing afferent presynaptic inhibition is via a channel-mediated depolarization of their intraspinal terminals which can be recorded extracellularly as a dorsal root potential (**DRP**). Based on measures of DRP latency it has been inferred that this primary afferent depolarization (**PAD**) of low-threshold afferents is mediated by minimally trisynaptic pathways with GABAergic interneurons forming last-order axoaxonic synapses onto afferent terminals. We used an in vitro rat spinal cord preparation under conditions that restrict synaptic transmission to test whether more direct low-threshold pathways can produce PAD. Mephenesin or high divalent cation solutions were used to limit oligosynaptic transmission. Recordings of synaptic currents in dorsal horn neurons and population synaptic potentials in ventral roots provided evidence that conventional transmission was chiefly restricted to monosynaptic actions. Under these conditions, DRP amplitude was largely unchanged but with faster time to peak and reduced duration. Similar results were obtained following stimulation of peripheral nerves. Even following near complete block of transmission with high Mg^{2+}/low Ca^{2+} containing solution, the evoked DRP was reduced but not blocked. In comparison, in nominally Ca^{2+}-free or EGTA-containing solution, the DRP was completely blocked confirming that Ca^{2+} entry mediated synaptic transmission is required for DRP genesis. Overall these results demonstrate that PAD of low-threshold primary afferents can occur by more direct synaptic mechanisms, including the possibility of direct negative-feedback or non-spiking dendroaxonic

pathways.

3.2 Introduction

The spinal cord receives a continuous barrage of sensory information that requires central mechanisms to limit and channel their signaling. A principal cellular mechanism for control of primary sensory afferents is via a depolarization of their terminals, termed primary afferent depolarization (PAD) which leads to a reduction in transmitter release. PAD can be measured experimentally following its antidromic electrotonic spread to dorsal roots as a dorsal root potential (DRP).

To date, electrophysiological experiments in the adult cat relying on measures of central latency have suggested that PAD of low-threshold cutaneous and muscle afferents is produced via a minimally tri-synaptic pathway involving last order GABAergic inhibitory interneurons (Rudomin and Schmidt 1999). In contrast, recent anatomical evidence demonstrated that only a single interneuron is interposed in some disynaptic circuits that could produce PAD of lower threshold afferents (Olave, Puri et al. 2002; Bannatyne, Liu et al. 2009). In addition, non-spiking spinal microcircuits in turtle have been shown to be sufficient to generate afferent-evoked PAD in TTX-insensitive high threshold afferents, further implicating more direct negative feedback mechanisms controlling afferent activity (Russo, Delgado-Lezama et al. 2000).

We designed experiments to provide the first electrophysiological evidence that lower-threshold afferents also evoke PAD by more direct synaptic pathways. We used the isolated hemisected juvenile rat spinal cord maintained in vitro and examined the effects of chemically-induced reductions in synaptic transmission on the DRP and on the excitability of spinal neurons. Our rationale was that if synaptic transmission could be limited principally to monosynaptic actions and the earliest DRP component is largely unaffected, then PAD evoked by stimulation of low threshold

afferents can be produced by more direct mechanisms. Indeed, the results presented suggest that more direct mechanisms, not mediated by trisynaptic or polysynaptic pathways, represent the predominant form of primary afferent stimulation evoked PAD.

3.3 Methods

All procedures were approved by the Emory University Institutional Animal Care and Use Committee. Midsagittally-hemisected spinal cords were isolated from Sprague–Dawley rats aged from postnatal days 7–14 and were prepared for in vitro experiments as described previously (Machacek and Hochman, 2006;Shay et al., 2005). Recordings were made in oxygenated aCSF containing (in mM): NaCl 128; KCl 1.9; D-glucose 10; $MgSO_4$1.3; $CaCl_2$ 2.4; KH_2PO_4 1.2; and $NaHCO_3$ 26.

The general experimental setup is shown in Figure 12. Dorsal lumbar roots (L3, L4 and/or L5) were left intact for stimulation of afferent fibers broadly separated by electrical stimulus intensity with bipolar glass suction electrodes. For some experiments, the tibial and deep peroneal hindlimb peripheral nerves were left intact for stimulation. Dorsal root potentials (**DRPs**) were recorded from cut dorsal root filaments or intact via *en passant* nerve suction. Ventral root potentials (**VRPs**) and accompanying reflexes were recorded from the ventral motor roots. Unless otherwise stated, recordings were made with differential amplifiers at DC with a gain of 1000 (AM Systems Model 3000) or near DC (high pass at 0.1 Hz; custom built with a gain of 10,000). Unless otherwise stated, constant current stimulators delivered single-shock stimuli every 30 seconds of defined stimulation intensity to the dorsal roots (usually L5) or peripheral nerves. Occasionally, a train of 5 pulses at 20 Hz was delivered 15 seconds after the single-shock stimulus. All actions are studied from large diameter/fast conducting myelinated low threshold afferents (cutaneous Aβ and muscle group I & II), typically at constant current stimulation values of 100μA, 100μs (Shay, Sawchuk et al. 2005) which equated to simulation at approximately 10

times the threshold for afferent fiber recruitment (T).In most experiments, the afferent volley was recorded antidromically distal to the site of stimulation to confirm selective recruitment of low-threshold afferents (Shay, Sawchuk et al. 2005) and to monitor volley amplitudes during drug applications to ensure observed actions are independent of changes in afferent fiber recruitment. This is critical in the current work as the solutions used to minimize synaptic transmission presumably do so by raising action potential threshold (Frankenhaeuser and Meves 1958; Ono, Fukuda et al. 1979). A suprathreshold stimulation intensity of 100 μA, 100 μs prevented such actions on afferent fiber recruitment and that resulting EFP. Whenever tested, the afferent volley and central actions were completely eliminated at low TTX dose (100 – 200 nM) supporting an absence of contribution from high threshold afferents (Fig 12).

To minimize di- and trisynaptic transmission, mephenesin (Sigma) at 1 mM or a high divalent cation aCSF containing 8 mM Mg^{2+}/ 4 mM Ca^{2+} was used (Jahr and Yoshioka 1986). To greatly diminish synaptic transmission a 6.5 mM Mg^{2+}/0.85 mM Ca^{2+} aCSF solution (Vyklicky, Sykova et al. 1976) or 100-500 μM EGTA were used. Bicuculline (10-20 μM), picrotoxin (25 μM) and gabazine (5 μM) (Sigma) were used to block transmission through $GABA_A$-like receptors. In all cases, control DRP responses were recorded for 1 hour, and the effects of drugs or solute exchange were recorded for a minimum of 30 minutes prior to washout and return to control aCSF.

3.3.1 Whole-cell patch clamp recordings.

Whole-cell patch-clamp recordings were made as detailed previously (Machacek and Hochman, 2006) to record evoked postsynaptic events after stimulation of attached roots. Patch electrodes contained (in mM); 140 K-gluconate,11 EGTA, 10 HEPES, 1 $CaCl_2$, and 35 KOH or 120 CsF, 10 EGTA, 10 HEPES, 10 CsCl2, 35 CsOH, and 5 QX314 (to block spiking) at pH7.3. Electrode resistances ranged from 2 to 6 MΩ. Recordings were obtained in conjunction with an Axopatch 1D amplifier filtered at 2 kHz (Molecular Devices, Union City, CA). Synaptic events were

recorded at the same membrane potential before, during, and after drug application. Recordings targeted the deep dorsal horn, the predominant termination site of low threshold primary afferents (Willis and Coggeshall 1991) and approximate location of putative interneurons interposed in PAD pathways (Jankowska, McCrea et al. 1981). Primary afferent stimulation evoked excitatory postsynaptic currents (**EPSCs**) were selected for and confirmed by the persistence of monosynaptic inward currents when the holding potential was raised to -40 mV. Monosynaptic currents were confirmed by latency to onset and onset consistency during 1 Hz stimulation (Bardoni, Torsney et al. 2004). Only neurons with clear monosynaptic EPSCs were included in the analysis.

3.3.2 Data Analysis

Data were acquired and analyzed on a personal computer using either pClamp software (version 10.0; Molecular Devices) or files were imported into MATLAB for analysis with a custom program. The last 5 sweeps of each recording were averaged 30 minutes after drug application or washout. Each sweep was 30 seconds in duration with a single stimulus pulse delivered at sweep onset. The resulting traces were then used to determine peak amplitudes, time to peak, duration, and area of DRPs and EPSCs. Values are reported as mean ± standard error. Student's t-test was employed to determine statistical significance. Unless otherwise stated, $p<0.05$ was considered as significant.

3.4 Results

3.4.1 PAD requires synaptic transmission, TTX-sensitive afferents, and is blocked by $GABA_A$ receptor antagonists.

At the stimulus intensity chosen we first demonstrated that the PAD measured with DRP recordings is generated by low-threshold afferents. Low-threshold afferent-evoked DRPs are sensitive to $GABA_A$ receptor antagonists, as shown previously (Rudomin and Schmidt 1999).

Here, we demonstrate that the DRP is abolished by the $GABA_A$ receptor antagonists bicuculline (n=13/13), picrotoxin (n=2/2), and gabazine (n=2/2; Fig 12B-D). To further confirm that we are looking at a low-threshold afferent fiber population, we applied low dose TTX (100-200 nM) on several occasions and always blocked the DRP and afferent volley (n=8/8) (Fig.12B).

While it is assumed that PAD is mediated by chemical synaptic transmission, this has not been tested in the mammal and may not apply to all afferent evoked responses (Vyklicky, Sykova et al. 1976). To demonstrate that chemical synaptic transmission is essential to the DRP generated following stimulation of low threshold afferents we perfused the cord in a nominally Ca^{2+}-free aCSF (n=4/4) or by applying the Ca^{2+} chelator EGTA (n=2/2). In both the heteronymous L4 dorsal root and the L5 ventral root the depolarizing potentials were completely abolished.

3.4.2 Mephenesin and high divalent cation solution largely restrict transmission to monosynaptic actions.

We explored whether PAD can be generated via more direct synaptic actions previously estimated to be minimally trisynaptic. To achieve this, we minimized synaptic transmission with bath applied mephenesin or exchanged the bath to a high divalent cation aCSF. At 10T or 100 μA, 100 μs, neither mephenesin nor high divalent cation solution had a significant depressant effect on the peak amplitude of the DRP produced by fast conducting afferent fibers (95±12% n=14 and 74±14% n=11 respectively). After restriction of synaptic actions by mephenesin or high divalent cation solution, the remaining DRP was still completely blocked by all $GABA_A$ receptor antagonists tested (n= 12) with examples for picrotoxin and gabazine illustrated in Fig 12C-D.

Extracellular field potentials (**EFPs**) evoked by afferent nerve stimulation predominantly reflect the population monosynaptic excitatory postsynaptic potentials of nearby neurons (Baldissera, Hultborn et al. 1981). EFPs were recorded in the deep dorsal horn. As shown in Figures 13Ai and Aii, stimuli delivered at 10T produced two field potentials corresponding to the two fast conducting low threshold afferent volleys activated at this stimulation intensity. The afferent

volleys and evoked monosynaptic field potentials were unaltered by mephenesin (Figure13Ai) and slightly altered by the high divalent cation solution (Figure 13Aii).

We next examined the effects of mephenesin and high divalent cation solution on longer latency synaptic actions. Concomitant recordings of ventral root reflexes including DC-coupled ventral root potentials (**VRPs**) were used to monitor population synaptic actions in motor neurons (Rudomin, Solodkin et al. 1987). Both mephenesin and the high divalent cation solutions isolated monosynaptic reflexes (n=5/5 and 3/3 respectively) and shorter latency, presumably monosynaptic sub threshold population VRPs (n= 10/10 and 6/6, respectively; Figure13Aiii). Combined the DC-coupled VRP duration was reduced to 66±11% of control values (n=9; $p<0.05$) due to a significantly reduced rise area (59±7% of control value; $p<0.01$) and decay area (60±11% of control; $p<0.01$), while the monosynaptic reflex amplitude was not significantly reduced (79.8±11.4% of control).Voltage clamp recordings from spinal neurons of afferent-evoked postsynaptic currents were used to provide further evidence of pharmacological block of polysynaptic actions. The deep dorsal horn was targeted as this is the site where PAD interneurons have been identified (Jankowska, McCrea et al. 1981) and where a large fraction of low-threshold primary afferents terminate (Willis and Coggeshall 1991). Intracellular recordings demonstrated a near-complete block of longer latency synaptic currents in the presence of mephenesin (n = 5/5) and a high divalent cation solution (n = 7/7; Figure13B). Monosynaptic actions remained and, where measurable, remained unchanged in peak amplitude. Note that the blocked longer-latency synaptic currents may appear to have latencies too long to be generated disynaptically. However, this is hard to ascertain since synaptic current onset cannot be measured due to the presence of an overlapping monosynaptic effect and conduction times of low threshold afferents at this age may lead to large latency delays (Shay, Sawchuk et al. 2005). Table 1B shows that for both mephenesin and high divalent cation-containing solution, the overall area of

decay was significantly reduced. In addition EPSC duration was significantly reduced overall to 48±15% for mephenesin and to 74±27% for a high divalent cation solution.

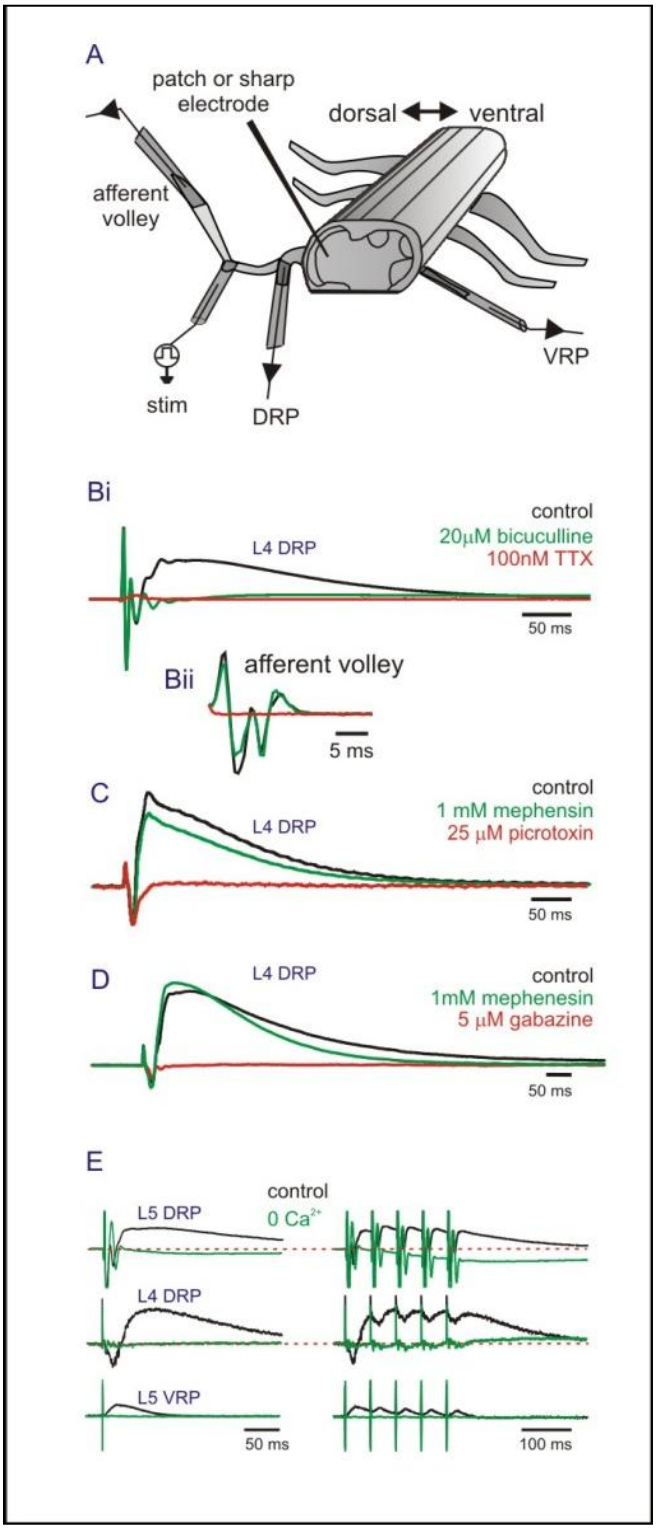
3.4.3 Bicuculline-sensitive DRPs remain after restricting synaptic transmission.

Overall, the above results support the use of mephenesin and high divalent cation solution to largely restrict transmission to monosynaptic actions. Critically, under these conditions, low threshold DRPs remained with 1 mM mephenesin (n = 14/14; Figures 12C, D, and 13Bii, Cii, D) or after replacement with a high divalent cation solution (8 mM Mg^{2+}/4 mM Ca^{2+}; n = 11/11; Figures13Aiii, Bi, Ci). DRP peak amplitude was not statistically different after application of these agents whereas average DRP duration was significantly reduced (from 471 ± 34 ms to 312 ± 71 ms; see also Table 1A). In addition, time to DRP peak was significantly reduced by mephenesin and the areas of DRP rise and decay were reduced for both agents (Table 1A).

Further comparison of mephenesin to the high divalent cation solution shows that both block later components of the DRP (Figure13C). In this example the high divalent cation solution also reduces the early component of the DRP.

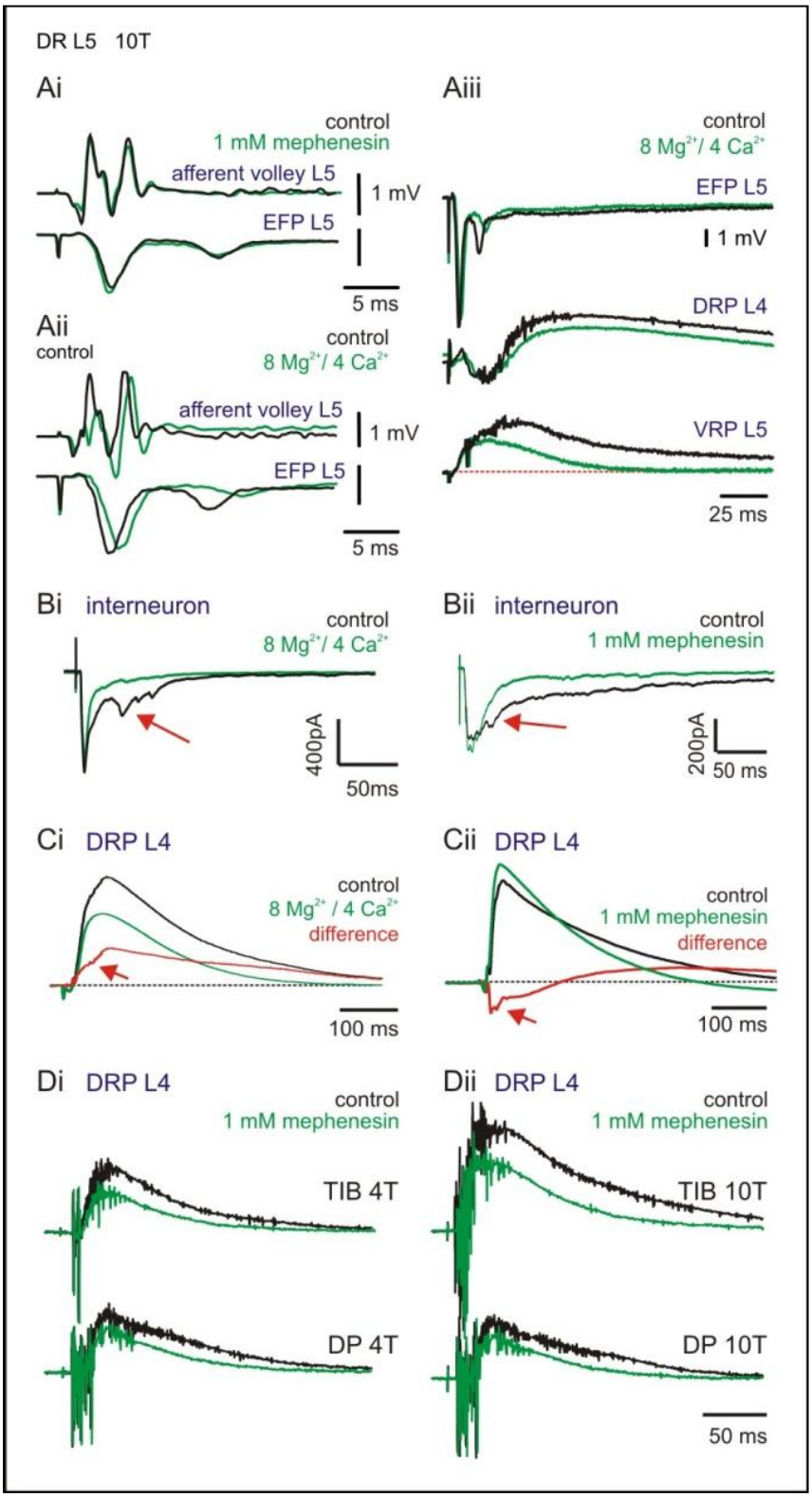
In a subset experiments (n=6), peripheral nerves were left in continuity with the spinal dorsal roots through which the afferents entered the lumbar cord (L4 and L5). The tibial and deep peroneal nerve were stimulated at 4T and 10T and at both stimulation intensities, mephenesin at 1 mM blocked only a small portion of the DRP (Figures 13Di and Dii). For tibial, DRP amplitude was reduced to 81% at 4T (n=4) and 69% of the original values at 10T (n=3). For deep peroneal these values are 76 and 81% respectively (n=3). In all instances short-latency EFP amplitudes were unchanged (not shown).

Figure 12 DRP is synaptically mediated and blocked by $GABA_AR$ antagonists



A. Experimental setup (see Methods for details). **B-D.** Stimulation is via the L5 dorsal root at 100 μA, 100 μs. **Bi.** A single stimulus produces a DRP that is almost entirely blocked by bicuculline. The remaining potential is TTX-sensitive. **Bii.** Low threshold activation is confirmed by measuring the afferent volley produced by this stimulation and its TTX sensitivity. **C.** Picrotoxin completely blocks DRP after synaptic isolation with mephenesin. **D.** Similarly, gabazine blocks the DRP after pre-incubation in mephenesin. **E.** The DRP requires chemical synaptic transmission. Evoked responses are shown before and after bath exchange to a nominally 0 Ca^{2+}-containing saline. Identical results were seen in another animal after application of the Ca^{2+} chelator EGTA (100 μM). Left column is a single shock and right column is five pulses at 20 Hz. *Traces were low pass filtered at 300Hz.*

Figure 13 Effects of mephenesin and a high divalent cation solution on the AV, EFP, EPSC and DRP.



Stimuli were delivered at 10T to the L5 dorsal root. Afferent volleys were recorded from the same dorsal root. Extracellular field potentials (EFPs) were recorded in the L5 deep dorsal horn. **Ai.** Stimulation recruits 2 separable afferent volleys, each leading to a monosynaptic field potential. These responses are unaffected by the presence of mephenesin. **Aii.** In the presence of a high divalent cation solution, the low threshold afferent volley waveforms and corresponding evoked field potentials are slowed. **Aiii.** Relation of monosynaptic EFPs to the evoked DRP and VRPs. The evoked DRP waveform shape is largely unchanged in the presence of the high divalent cation solution whereas VRP duration is greatly shortened. **Bi & Bii.** EPSCs in 2 neurons measured at a holding potential of -80 mV. Identity of synaptic current was verified as excitatory at a holding potential of -40 mV. Under both conditions (high Mg^{2+}/high Ca^{2+} and mephenesin) the longer-latency synaptic response (at arrow) is blocked while the monosynaptic component is not significantly affected. **Ci & Cii.** High divalent cation solution and mephenesin both block longer latency DRPs in the same animal (arrows on difference trace identifying inflection of longer-latency component). **D.** Similarly, most of the DRP remains after treatment with mephenesin following stimulation of the tibial or deep peroneal nerve at 4T (**Di**) or 10T (**Dii**). *Traces were low pass filtered at 3 kHz and at 300 Hz in panel C.*

3.4.4 Further evidence of minimal synaptic transmission requirements in afferent-evoked PAD.

We used a 6.5 mM Mg^{2+}/0.85 mM Ca^{2+} solution to severely minimize synaptic transmission (Vyklicky et al., 1976). Under these conditions the DRP is either almost completely blocked (heteronymous root; not shown) or a small component remains (homonymous root) (n = 4/4; 10 ± 4% of control; Figure14Ai). However, following repetitive stimulation with 5 pulses at 20 Hz, the heteronymous DRP re-emerges (not shown) and the homonymous DRP facilitates. This demonstrates that even with only minimal chemical synaptic transmission, a low-threshold DRP is evoked via more direct actions of afferents on their terminals. Recordings from monosynaptically-connected interneurons show a nearly identical phenomenon where repetitive stimulation is required in order to observe postsynaptic currents in these interneurons (n = 4/4; Figure14Aii).

3.5 Discussion

The data in this chapter challenge the assumption, based exclusively on latency measures, that PAD predominantly involves a minimally trisynaptic pathway involving excitatory and GABAergic interneurons (Eccles, Magni et al. 1962; Jankowska, McCrea et al. 1981; Rudomin and Schmidt 1999). After applying solutions thought to block all but monosynaptic transmission, the evoked DRP was not significantly diminished in amplitude (Table 1A). This is consistent with an earlier *in vivo* report on the actions of mephenesin (Farkas, Tarnawa et al. 1989). Evidence of synaptic isolation was corroborated with intracellular recordings in dorsal horn neurons showing block of longer latency excitatory currents and reduced duration of population synaptic actions in motor neurons by recording their electrotonic spread as a VRP. VRPs and EFPs monitor population responses confirming a decrease in polysynaptic actions after application of mephenesin or a high divalent cation solution. We verified that the afferent fiber population recruited was the from fast conducting, TTX-sensitive fibers. The evoked DRP was

blocked using $GABA_A$ receptor antagonists, demonstrating that the DRP involves activation of $GABA_A$ or $GABA_A$-like receptors. Even stronger evidence for PAD being produced by more direct mechanisms was demonstrated by its expression even under conditions of near-complete block of synaptic transmission with a high Mg^{2+}/low Ca^{2+} aCSF.

The most conservative interpretation of our findings is that PAD of many low-threshold afferents does not require trisynaptic pathways but does utilize di- or even mono-synaptic axoaxonic pathways. In the case of a disynaptic pathway, PAD-producing interneurons are still recruited during mephenesin or high-divalent cation solution induced reductions in transmission. While probably a component, di- and polysynaptic pathways are unlikely the dominant mechanism for the early DRP as its amplitude was only slightly depressed when longer latency synaptic actions (EPSCs and VRPs) were largely abolished.

The identity of the low-threshold afferents by which more direct pathways produce PAD requires future investigation. For example, PAD generated by some afferents may require minimally trisynaptic pathways on some afferents but may generate more direct negative feedback onto homonymous terminals (Wall 1958; Olave, Puri et al. 2002; Bannatyne, Liu et al. 2009). Regardless, given that most of the DRP remains after synaptic transmission reduction, it seems likely that many low-threshold afferents produce PAD by more direct circuits.

3.5.1. Is PAD generated monosynaptically or via unconventional non-spiking microcircuits?

The most straightforward interpretation of the data is that a major component of the DRP is generated by direct monosynaptic or extrasynaptic negative feedback mechanisms. Direct feedback would require that primary afferents release transmitter(s) acting on $GABA_A$ or $GABA_A$-like receptors, but numerous labeling studies have failed to support GABA as a transmitter localized in primary afferents (Todd, 1996). Alternate candidate amino acids are taurine and β-alanine, as both activate $GABA_A$ receptors to produce PAD (Nicoll and Alger

1979). Microdialysis experiments demonstrate taurine and β-alanine release following activation of primary afferents by as much as glutamate (Paleckova, Palecek et al. 1992). There is also evidence of taurine immunoreactive primary afferents (Lee, Renno et al. 1992).

Another possibility is that acetylcholine (ACh) is co-released from primary afferents to act on bicuculline-sensitive nicotinic receptors. Peripheral choline acetyltransferase (pChAT) is found in both small and large diameter primary afferents (Bellier and Kimura 2007) and immunolabeling studies identify pChAT in myelinated dorsal column primary afferents (Yasuhara, Aimi et al. 2008). Large-diameter afferents preferentially express the vesicular ACh transporter as well as acetylcholinesterase (Willis and Coggeshall 1991; Tata, De Stefano et al. 2004; Bellier and Kimura 2007). Primary afferents also contain bicuculline-sensitive α9 and α10 nicotinic ACh receptor subunits (Rothlin, Katz et al. 1999; Lips, Pfeil et al. 2002; Alexander, Mathie et al. 2007) and preliminary results demonstrate reduction in DRP amplitude by nicotinic receptor antagonists with reported actions on α9 receptors (Hochman, Shreckengost et al. 2010).

It is also possible that PAD is produced by disynaptic dendroaxonic pathways. In turtle, a non-spiking microcircuit generates a DRP evoked following stimulation of high-threshold TTX-insensitive fibers (Russo et al., 2000). Primary afferents contain numerous dendroaxonic synapses (Alvarez 1998) so it is conceivable that microcircuits based on these synapses produce PAD at various low-threshold afferents and these actions would be resistant to mephenesin and high divalent cation solutions. This would also be consistent with DRPs seen under conditions of minimal transmission where postsynaptic spiking would not be required.

Figure 14B summarizes these possibilities.

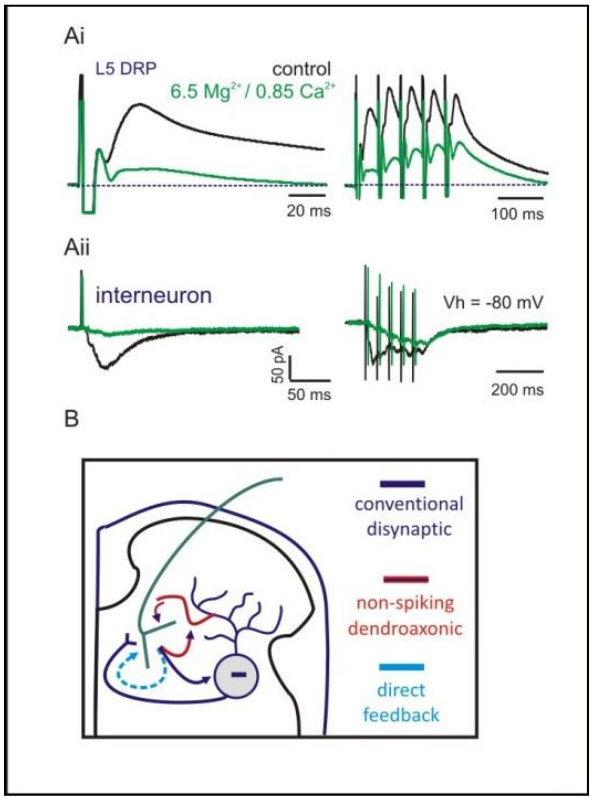
Table 1Effects of 1 mM mephenesin- and high divalent cation-containing solutions on dorsal root potential and excitatory postsynaptic currents.

A. Dorsal Root Potentials		Peak Amplitude (% control)	Time to Peak (% control)	DRP Duration (% control)	Area of rise (% control)	Area of decay (% control)
Mephenesin (1 mM)	n=14	94.7±11.7	81.7±5.2**	66.6±3.8**	77.35±8.7*	69.0±8.2**
Wash	n=8	109.7±30.0	102.1±9.2	75.0±8.4*	120.7±28.4	90.8±24.9
High Mg^{2+}/High Ca^{2+}	n=11	74.0±14.4	109.7±14.5	74.6±4.0**	61.3±10.5**	54.1±10.1**
Wash	n=8	76.0±15.6	113.6±9.3	90.8±4.2	96.8±17.5	71.4±15.5
B. EPSCs		Peak Amplitude (% control)	Time to Peak (% control)	Duration (% control)	Area of rise (% control)	Area of decay (% control)
Mephenesin (1 mM)	n=4	114.1±37.7	114.8±13.7	48.3±14.9*	94.9±18.6	21.1±4.6**
Wash	n=4	110.6±32.8	129.4±33.7	71.0±23.0	94.5±16.1	36.7±14.7*
High Mg^{2+}/High Ca^{2+}	n=7	66.2±20.4	81.6±12.5	73.8±27.7*	60.5±18.4	23.8±3.6**
Wash	n=6	98.9±25.0	104.9±9.9	97.6±50.2	86.1±17.9	52.1±14.5*

Values represent percent of control reported as mean ± standard error. Time to peak was measured from where the signal crosses baseline to the peak of the DRP. DRP or EPSC duration is the measure of the signal crossing baseline to the point at which the DRP decayed to 10% of

the peak value. Area of rise represents the area under the curve from where the signal crosses baseline to the time of peak amplitude. Area of decay represents the area under the curve from the time of peak amplitude to the time point where the signal decays to 10% of the peak value. *, $P<0.05$; **, $P<0.01$.

Figure 14 The DRP remains after block of synaptic transmission and proposed mechanisms



A. Greatly reducing synaptic transmission does not completely block the DRP. Evoked responses are shown before and after bath exchange to a high Mg^{2+} / low Ca^{2+} saline (6.5 mM Mg^{2+}/0.85 mM Ca^{2+}). Stimulation is via the L5 dorsal root at 100 μA, 100 μs. **Ai.** Left, Single shock; right, five pulses at 20 Hz. In high Mg^{2+}/low Ca^{2+} saline, the DRP is facilitated following repetitive stimulation. **Aii.** Under the same conditions in another animal, the high Mg^{2+}/low Ca^{2+} saline virtually abolished monosynaptic excitatory postsynaptic currents, but transmission is increased by repetitive stimulation (upper row). *Traces were low pass filtered at 300 Hz.* **B.** Putative circuits for low-threshold afferent-evoked PAD not requiring trisynaptic pathways. As mephenesin and high divalent cation solutions block part of the DRP, a conventional disynaptic pathway may serve PAD of some primary afferents. The remaining DRP may arise from direct monosynaptic actions of transmitter onto afferent terminals or via a disynaptic non-spiking dendroaxonic microcircuit. For details see Discussion.

CHAPTER 4 EFFECTS OF CHOLINERGIC TRANSMISSION AND RECEPTOR ACTIVATION ON PRIMARY AFFERENT DEPOLARIZATION

4.1 Abstract

Chapter Two introduced the complex pharmacology associated with afferent evoked primary afferent depolarization (PAD) with the suggested involvement of cholinergic transmission onto and from primary afferents. In this chapter I undertook a detailed pharmacological analysis of cholinergic transmission in DRP producing pathways as well as an examination of the nicotinic receptors expressed on primary afferents using immunohistochemistry. I show that nicotinic acetylcholine receptors are integrated into the circuit for producing the afferent-evoked dorsal root potential (DRP) in cutaneous afferents. In particular, α9-containing nicotinic acetylcholine receptors (nAChRs) appear to be involved in transmission from slowly conducting A-fibers and the production of the DRP. I show that α9 receptors are expressed in a subpopulation of dorsal root ganglion (DRG) neurons; largely in afferents with medium diameter cell bodies. Additionally, I describe the phenomenon of direct depolarization of afferents through activation of nAChRs located directly along afferent axons. A direct DC depolarization of primary afferents is mediated by activation of α3, α6, α7 and α9 containing nicotinic receptors. These receptors are located on subsets of afferents along axons, ranging from the dorsal root to the periphery in both muscle and cutaneous afferents. When activated, these receptors can directly depolarize afferents and can affect afferent excitability. The discrepancy in the pharmacology of these two phenomena suggests that they are distinct physiological processes, one involving nAChRs in production of the DRP, and the other leading to direct afferent depolarization through activation of nAChR situated along afferent axons. Thus, I demonstrate that ACh and nAChRs contribute to two separate forms of controlling sensory transmission in primary afferents.

4.2 Introduction

In Chapter 2, I demonstrated that DRPs have an unusual pharmacology and that ACh could be involved in production of a portion of the DRP. I also show evidence that ACh may be released from primary afferents, outlining a putative direct mechanism through which DRPs could be produced. Chapter 3 focused on the production of this more 'direct' DRP, showing that the presumed trisynaptic pathway is not the sole mechanism for DRP production. This chapter examines the contribution of ACh and nicotinic ACh receptors to production of the DRP. I also investigate the phenomenon of afferent depolarization through activation of nicotinic ACh receptors located directly on primary afferent axons in the periphery. I demonstrate that α9 nicotinic receptors play a role in transmission from slower conducting A-fibers in production of the DRP. I also show that α3, α6, and α9 containing nicotinic ACh receptors are located of afferent axons in the periphery and that activation of these receptors leads to afferent depolarization. Finally, I present evidence that α7 nAChRs can affect afferent excitability.

4.2.1 Is the acetylcholine transmitter system involved in afferent-evoked PAD?

Below, I outline previous evidence suggesting that the acetylcholine transmitter system participates in PAD, either via cholinergic interneurons or via direct release from primary afferents themselves. The former is the more likely explanation for cholinergic involvement in the DRP, as I have shown in Chapter 2 that block of glutamatergic transmission can block the DRP. However, direct release of ACh from primary afferents may still play a role in modulation of afferent signaling through production of dorsal root reflexes, which will be discussed in greater detail below.

Lamina III is a projection site of Aβ and Aδ cutaneous afferents (Willis 1991) and the likely location of interneurons interposed in PAD of cutaneous afferents, based on interneuron firing

coincident with DRP onset (Jankowska, McCrea et al. 1981). It is also the predominant location of dorsal horn cholinergic interneurons (Barber, Phelps et al. 1984), placing them in a relevant spatial context to contribute to PAD (Ribeiro-da-Silva and Cuello 1990; Todd 1991; Alvarez 1998). A dense cholinergic plexus of cholinergic fibers in lamina II/III is composed of cholinergic dendrites contacted by primary afferents and cholinergic terminals for axo-axonic connections onto primary afferents (Barber, Phelps et al. 1984; Ribeiro-da-Silva and Cuello 1990; Alvarez 1998). Recent studies have also demonstrated that cholinergic interneurons with cell bodies in lamina III show repetitive firing patterns following current injection supporting possible involvement in a long lasting phenomenon such as the DRP (Mesnage, Gaillard et al. 2011). ACh and GABA co-exist in a population of dorsal horn interneurons and at presynaptic terminals to primary afferents (Todd 1991). ACh is found in 25% of GABAergic interneuron terminals presynaptic to primary afferent axons (Alvarez 1998). Thus, ACh interneurons could participate in disynaptic actions onto primary afferent terminals, and I will show that cholinergic transmission onto primary afferents can affect both DRP generation and transmission from slower-conducting A-fibers through α9 nAChRs.

4.2.1.2 Acetylcholine release from primary afferents.

In Chapter 2, I present evidence for the possibility of ACh release directly from primary afferents. To date, no studies have demonstrated ACh release from primary afferents. However, with all of the mechanisms to synthesize, package, and degrade ACh, localized to primary afferents, it is likely that release of ACh direct from afferents is occurring. However, one unexplained feature of pChAT staining is that it does not appear to be found at synaptic terminals, even though expression in the cell bodies has been widely reported (Bellier and Kimura 2007; Matsumoto, Xie et al. 2007; Mesnage, Gaillard et al. 2011). Further examination with more sensitive detection techniques, such as electron microscopy, may challenge this view. If pChAT is absent in terminals, an intriguing possibility is that ACh is released extrasynaptically at axonal branch

points or along the length of the primary afferent axons to control the level of PAD via direct feedback. Presynaptic inhibition via actions at branch points is well-documented in invertebrates (Smith 1980) and has also been demonstrated in mammalian afferents (Verdier, Lund et al. 2003). Here, I present evidence that activation of α3, α6, and α9 nAChRs located along afferent axons in the periphery can lead to a direct depolarization of primary afferents.

4.2.2 Nicotinic receptors are expressed in primary afferents

Primary afferents contain numerous nicotinic receptor subtypes (Genzen, Van Cleve et al. 2001; Lips, Pfeil et al. 2002) that could be responsible for a portion of bicuculline-sensitive PAD. Bicuculline, which is known to reduce PAD in primary afferents potently inhibits the α9 homomeric nAChR receptors (IC_{50}=0.8 μM) (Rothlin, Katz et al. 1999; Demuro, Palma et al. 2001), α9,α10 heteromeric nAChRs (Alexander, Mathie et al. 2007), and α7 and α4-containing nAChRs (Demuro, Palma et al. 2001), which are also found in DRG neurons (Boyd, Jacob et al. 1991; Lips, Pfeil et al. 2002; Hancock, Canetta et al. 2008), raising the possibility that part of PAD is nicotinic receptor-mediated. Critically, medium to large diameter DRG neurons contain α-bungarotoxin binding sites, and thus nAChRs (examined in rat, cat, monkey, and human) (Ninkovic and Hunt 1983). These neurons represent at least 12% of large diameter afferents and appear to project selectively to lamina III, the projection site of Aβ cutaneous afferents (Willis 1991) and presumably the location of interneurons interposed in PAD of cutaneous afferents (Jankowska, McCrea et al. 1981). Furthermore, after removal of primary afferent innervations through dorsal root rhizotomy, α-bungarotoxin binding *specifically* decreases in lamina III, and nowhere else in the spinal cord (Ninkovic and Hunt 1983), suggesting that the primary afferents projecting here are expressing ACh receptors.

4.2.3 ACh acts on ionotropic and metabotropic ACh receptors

Acetylcholine acts on two classes of receptors, ionotropic and metabotropic. The ionotropic receptors are referred to as nicotinic acetylcholine receptors (nAChRs), due to the ability of

nicotine to activate many of these receptor subtypes. Nicotinic acetylcholine receptors are composed of α and β subunits that form pentameric ligand-gated cation channels. Nine α subunits (α2–α10) and three β subunits (β2–β4) have been identified in vertebrate neuronal tissues, and different combinations of these subunits define nAChR subtypes. While all nAChRs receptors conduct Na^+, K^+, and Ca^{2+} when activated, the different receptor subtypes have distinguishing pharmacological and/or functional properties related to subunit assembly, including binding affinities of ligands, efficacies and potencies of agonists and antagonists, channel conductances, and rates of desensitization and recovery (Lindstrom et al., 1996; Albuquerque et al., 1997; Colquhoun and Patrick, 1997). Importantly, α7, α9 and, α10 nAChRs conduct Ca^{2+} to a much greater extent than the other nAChRs (Seguela, Wadiche et al. 1993; Gong, Zhou et al. 2003), and the presence of these receptors on primary afferent may have a much greater or unique effect on primary afferent transmission than causing PAD, such as increasing the probability of release or actually causing release from afferent terminals. The relative permeability of calcium to sodium ions is ~2:1 for heteromeric neuronal non-α7 nAChRs, and >10:1 for homomeric α7 and α9 receptors or the heteromeric α9/α10 nAChRs (Shen and Yakel 2009).

As nAChRs are cation conducting channels, their reversal potential is ~0 mV (or higher if predominantly Ca^{2+} conducting), which has implications for their action on primary afferents. As alluded to earlier, PAD is generally thought to be mediated by activation of $GABA_A$Rs, which leads to a depolarization in primary afferents, as the reversal potential for GABA is -26 mV, as determined in DRG somata in cat (Gallagher, Higashi et al. 1978). The enhanced driving force of nAChRs suggests that their activation will lead not only to depolarization of the afferents but also to an increased probability of spike generation in afferents (referred to as dorsal root reflexes or DRRs). The reversal potentials of $GABA_A$ and nACh receptors, along with their ionic conductances suggests distinct actions on primary afferents, for though PAD has limited range of electrotonic spread in afferent axons dependent on the axonal length constant, DRRs propagate

both bi-directionally along the entire length of the axon, implicating peripheral as well as central actions. nAChRs may thus play a large role in controlling afferent excitability and spike generation.

Additionally, ACh activates metabotropic receptors, known as muscarinic receptors, named due to the affinity of muscarine for these receptors. There are 5 different types of muscarinic receptors (M_1 - M_5), and most tissues express a mixture of subtypes (Brown 2010).

The purpose of the present study was to examine the cholinergic mechanisms contributing to production of the DRP along with direct depolarization of primary afferent through nAChRs. Using multiple drugs to determine the subunit composition of the nAChRs involved in these phenomena and a preparation that allowed for stimulation and recording from isolated peripheral cutaneous afferents, I investigated nicotinic involvement in sensory transmission from primary afferents. Coupled with immunohistochemistry to support physiological observations, I show that α9 receptors are involved in transmission from slower conducting A-fibers and DRP production from dorsal root and cutaneous nerve stimulation. Additionally, I demonstrate direct DC depolarization of primary afferents through α3, α6, and α9 containing nAChRs, and that α7 nAChRs contribute to afferent excitability. These findings offer the possibility of future translational studies targeting α3, α6, and α9 nAChRs as a way to control aberrant sensory signaling in primary afferents in conditions such as spinal cord injury or neuropathic pain.

4.3 Methods

All procedures were approved by the Emory University Institutional Animal Care and Use Committee. Midsagittally-hemisected spinal cords were isolated from Sprague–Dawley rats aged from postnatal days 7-12 and were prepared for in vitro experiments as described previously (Machacek and Hochman, 2006;Shay et al., 2005) or from mice of the same age from the either the FVB or balb/c background. Mouse was chosen for the majority of experiments as a basis for future studies employing transgenic animals for selective labeling of afferent populations for intracellular recordings, examination of the effects of deletion of nicotinic receptor subtypes, as well as activation of afferent subpopulations through targeted expression of light sensitive ion channels. Recordings were made in oxygenated aCSF containing (in mM): NaCl 128; KCl 1.9; D-glucose 10; $MgSO_4$1.3; $CaCl_2$ 2.4; KH_2PO_4 1.2; and $NaHCO_3$ 26.

4.3.1 Overview of sensory afferent classes

Sensory afferents arising from skin or muscle can be broadly separated into two categories - low-threshold and high-threshold. In general, low-threshold afferents from muscle and skin convey information about proprioception or touch, while most high-threshold fibers convey information regarding temperature and pain.

As will be demonstrated in Section 4.3.3. below, it is clear that the classical definition of threshold and separation of afferent fibers does not apply to the neonatal rodents (Vejsada, Palecek et al. 1985; Fitzgerald 1987) as Aβ/I/II are not clearly distinguishable from Aδ/III afferents leading to analyses that can only separate myelinated (Aβ/Aδ/I/II/II) from unmyelinated (C/IV) afferents.

4.3.2 Isolation and recording of dorsal roots and peripheral nerves

The general experimental setup is shown in Figure 15A with example traces that identify components of recorded potentials. In some experiments, dorsal lumbar roots (L3, L4 and/or L5)

were used for stimulation and recording. In other experiments, the tibial (Tib), superficial peroneal (SP), and sural (Su) hindlimb peripheral nerves were left intact for stimulation and recording. SP and Su contain solely cutaneous afferent information, and Tib is a mixed afferent nerve. The sensory afferents in Tib innervate the plantar surface of the foot, along with some intrinsic muscles of the foot. Su innervates the skin on the posterolateral portion of the lower hindlimb, the skin and fascia of the ankle, and the lateral side of the fifth digit. SP innervates the skin of the anterolateral portion of the hindlimb, the medial and distal regions of the foot, the medial distal surface of the first digit, and the interdigit skin of the remaining digits (Greene 1935).

PAD was inferred from antidromically propagating DRPs recorded at L3 through L5 dorsal roots by means of bipolar glass suction electrodes (~120 μm of tip internal diameter) placed en passant on the dorsal root as close as possible to the entry zone. The shortest-latency spiking components found in these dorsal root recordings correspond to the orthodromic afferent volley (AV) of lower threshold afferents.

Recordings of DRPs and AVs were made with differential amplifiers at DC with a gain of 1000 (AM Systems Model 3000 or custom built optically isolated amplifier [Bill Goolsby, Emory University]) or near DC (high pass at 0.1 Hz, low pass at 3 kHz; custom built amplifiers with a gain of 10,000). DC recordings were performed while dorsal roots or peripheral nerves were concurrently stimulated, so that the polarization state of the afferents could be monitored during drug application simultaneously with stimulus evoked effects. Stimulation and recording were both performed with bipolar glass suction electrodes.

4.3.3 Stimulation of peripheral nerves and dorsal roots

Constant current stimulators delivered single-shock stimuli every 15 – 30 seconds of defined stimulation intensity to the dorsal roots (usually L5) or peripheral nerves. When noted, peripheral nerves were dissected out and stimulated independently. Resulting DRPs were recorded from the dorsal root entry zone of a dorsal lumbar root (L3, L4, or L5), where the stimulated afferents enter the spinal cord, as is evidenced by the appearance of the AV preceding the evoked DRP (Figure 15B). Results from stimulation of peripheral nerves and dorsal roots were often combined for analysis. The reasoning for this was two-fold. While A-fiber muscle afferents have a lower threshold for recruitment than cutaneous A-fibers, a smaller amount of current is required to elicit measurable DRPs in cutaneous afferents. Secondly, the DRP elicited from cutaneous afferents are much larger than those elicited by muscle afferents at the same stimulus intensity (data not shown). Therefore, when stimulating the dorsal root, cutaneous afferent-evoked DRPs likely dominate the dorsal root-evoked DRP. Additionally, very few differences were seen in the pharmacology of dorsal root and cutaneous afferent evoked events.

Some experiments defined stimulus intensity by the threshold (T) of the most excitable afferents, as measured by the evoked AV, and stimuli were delivered in multiples of this intensity (2T, 4T, 10T, etc.) for 200-500 μs. When this was done, fiber recruitment was determined based on the stimulus intensities required to elicit A- and C-fiber components of the AV in the dorsal root or sciatic nerve (Figures 16 and 17A, bottom panel). DRPs and EFPs were observed for faster conducting myelinated afferents (Aβ and Aδ), and slowly conducting, unmyelinated afferents (C). These 2 broad categories were readily distinguished by the components of the AV produced by a given stimulus. This is exemplified in Figure 16 for stimulating of the tibial nerve while recording in the sciatic nerve. Progressively increasing stimulus intensities (both constant current and multiples of threshold) corresponded to differential fiber recruitment as monitored in the sciatic nerve AV. Even as low as 4T and 50 μA, 50 μs, a C-fiber component could be distinguished in

the afferent volley. The C-fiber volley increases with increasing stimulus intensity and a depression of the A-fiber component is also observed at higher stimulus intensities. I interpret the longer latency AV as representing unmyelinated afferents, as no additional later-arriving volley was recruited at supramaximal stimulation intensities (1 mA, 1 ms). Figure 17 shows evoked responses following stimulation of a purely cutaneous nerve. In Figure 17A, stimulation of Su at 20 μA, 50 μs shows no evidence of a C-fiber volley. However, increasing the stimulus intensity to 100 μA, 100 μs evokes a robust C-fiber volley. The components of the AV identified as A- and C-fiber mediated coincide with separable components of the dorsal root potential (DRP; Figure 17A). Likewise, stimulation at 20 μA, 50 μs recruits the shortest latency component of the EFP. Occasionally at 100 μA, 100 μs, a longer latency component of the EFP enters (Figure 17A; arrow), presumably a result of C-fiber recruitment. Observation of longer latency components is not often the case, as EFPs are recorded from an area of maximal potentials from A-fiber cutaneous stimulation (deep dorsal horn) and C-fibers generally terminate in more superficial regions of the dorsal horn.

Stimulation of cutaneous afferents at a strength that recruits both A- and C-fiber components of the DRP allow us to observe preferential drug actions on these fiber groups (components identified in Figure 17B). For experiments comparing A- and C-fiber mediated components of the DRP, the stimulation intensity was regularly 100 μA, 100 μs for peripheral nerves containing purely cutaneous afferents.

4.3.4 Dorsal Root Reflexes

When primary afferent depolarization brings the membrane potential of the afferent above threshold for action potential generation, antidromic spikes are seen in the dorsal root recordings. These are called dorsal root reflexes (DRRs; Figure 17C, top panel, arrow). DRRs were generally monitored at the dorsal root entry zone though they were also seen in recordings further distally along the dorsal root (Figure 17C, bottom panel) and also in recordings of peripheral nerves (data

not shown). These were differentiated from the AV by variable spike timing and shape following stimulation. Actions on DRRs without an effect on the evoked DRP would provide evidence that spiking and PAD in primary afferents are produced by different mechanisms and that they may also differ in their physiologic function.

4.3.5 Extracellular Field Potentials

Extracellular field potentials (EFPs) reflect the postsynaptic transmembrane currents evoked in the population of neurons around the tip of the electrode (Figure 17D&E) (Mesnage, Gaillard et al. 2011). Thus, they provided a measure of monosynaptic transmission from primary afferents onto first order spinal interneurons. EFPs were monitored to look at population effects of afferent stimulation on first-order spinal interneurons in order to determine if drug effects were due to actions on receptors located on primary afferent or on their post-synaptic targets.

EFP recordings were made using 1.5 mm outer diameter capillary tubes pulled with a Narashige electrode puller to a final tip diameter of 2-3 µm. Electrodes were then filled with a 2 M NaCl solution and resistances ranges from 4-6 MΩ. EFPs were amplified with an Axoclamp-1D (Axon Instruments, Molecular Devices, U.S.A.) at a gain of 3,000X and low pass filtered at 2000Hz.

Electrodes were positioned in the deep dorsal horn with the electrode entering the cord at a 90 degree angle to the midline at the interface between the dorsal column and the gray matter and going to depths 80-120 µm below the cut surface of the cord, where the largest responses were seen after stimulation of peripheral cutaneous nerves (Figure 17D) or the dorsal root (Figure 17E). The region or maximal EFPs was taken to be in the deep dorsal horn as this is where low-threshold cutaneous afferents terminate, which I identified by incremental tracking through the dorsal horn until the area with the largest cutaneous afferent-evoked EFP was observed when stimulating at A-fiber strength. Both short fast-rising fast-decaying as well as a longer-latency slowly decaying component were observable. Stimulation at C-fiber strength occasionally added

another component to the EFP (Figure 17A, arrow), but this was not always the case. While the peak of the EFP is a good indication of the strength of the afferent input onto spinal interneurons, the shape of the field potential was not always regular, so the area of the field also measured. Finally, I also calculated the initial slope of the EFP, as the slope is commonly used as a measure of synaptic strength (Abraham, Gustafsson et al. 1987), because peak amplitude, but not initial slope, can be affected by spikes occurring at the peak of the EFP. While EFPs provide information about the synaptic strength of monosynaptic transmission of primary afferents onto spinal interneurons, one cannot determine whether changes in the area of the EFP occur by either pre- or post-synaptic mechanisms on interneurons.

4.3.6 Drugs

Drug effects, particularly the cholinergic agonists, often took between 30 and 60 minutes to fully wash out and for the recorded signals to recover. As such, subsequent applications of all drugs were only done after the preparation had washed in regular aCSF for 1 hour. Accordingly, the washout amplitudes reported are those measured at least one hour after the drug was no longer in the perfusate.

The afferent volley, in addition to confirmation of A- or C-fiber recruitment was monitored to ensure that drug actions were due to actions on receptors mediating transmission, and not on propagation of the signal along the afferent axon.

Below is a list of the drugs used. For each I report their source of purchase, reported receptor pharmacology and dose used in experiments. Drug dosage was chosen based on several factors. In general, doses were determined based on published studies at concentrations that showed full block at a given receptor's actions in an intact *in vitro* or slice preparation. When these values could not be found, doses at 10x the reported IC_{50} value of a drug on receptors in culture expression systems was applied. However, if drugs were shown to have non-specific effects at

other, non-nicotinic receptors (i.e. d-tubocurarine at $GABA_A$Rs), I chose doses that led to maximal block of nicotinic receptors without actions at non-specific sites.

4.3.6.1 Muscarinic Receptor Antagonists

4.3.6.1.1 Atropine

Atropine [Sigma] is a non-selective antagonist at all muscarinic receptors (Caulfield and Birdsall 1998). Atropine was always applied at 10 μM. Atropine has previously been shown to block muscarinic actions in the mouse spinal cord. Atropine is typically applied at concentrations of 1-10 μM in spinal slice physiological experiments to completely eliminate and neuronal actions via muscarinic acetylcholine receptors (Cordero-Erausquin and Changeux 2001; Ziskind-Conhaim, Wu et al. 2008). Due to the use of the isolated, hemisected spinal cord preparation, we used 10 μM to ensure adequate penetration to deeper laminae within the spinal cord. Atropine was dissolved in H_2O to a stock concentration of 10 mM.

4.3.6.2 Nicotinic Receptor Antagonists

4.3.6.2.1 d-tubocurarine

D-tubocurarine Chloride Hydrate [Sigma] is a broad spectrum nAChR competitive antagonist that also has some properties of an open-channel blocker at α1, α2, α3, α4, α6, α7, α9, and α10 subunit containing nAChRs (Alexander, Mathie et al. 2007). D-tubocurarine was dissolved in H_2O to a stock concentration of 100 mM and applied at 10-30 μM, unless otherwise stated.

4.3.6.2.2 Mecamylamine

Mecamylamine Hydrochloride [Sigma] is a non-competitive open-channel blocker at α3, α6 and α9-containing nAChRs (Verbitsky, Rothlin et al. 2000; Alexander, Mathie et al. 2007). Mecamylamine was dissolved in H_2O to a stock concentration of 50 mM and applied at 50 μM.

4.3.6.2.3 Dihydro-β-erythrodine (DHβE)

DHβE hydrobromide [Tocris] is a competitive antagonist at α2, α3, and α4-containing nAChRs (Chavez-Noriega, Gillespie et al. 2000; Alexander, Mathie et al. 2007). DHβE was dissolved in H_2O to a stock concentration of 50 mM and applied at 50 μM.

4.3.6.2.4 Methyllycaconitine (MLA)

Methyllycaconotine citrate [Tocris] is generally considered to be a specific α7 nAChR antagonist (Alkondon, Pereira et al. 1992; Alkondon, Pereira et al. 1998), though antagonist actions have been reported at α3/α6-containing nAChRs (Mogg, Whiteaker et al. 2002). Methyllycaconitine was dissolved in H_2O to a stock concentration of 1 mM and applied at 0.2-1 μM.

4.3.6.2.5 α-bungarotoxin

α-bungarotoxin [Biotium, Inc] is an irreversible competitive antagonist at α1 (non-neuronal) and α7 nAChRs and a competitive antagonist at α9 and α10 subunit-containing nAChRs (Albert, Eccleston et al. 1959; Alexander, Mathie et al. 2007). α-bungarotoxin was dissolved in H_2O to a stock concentration of 100 μM and applied at 100 nM.

4.3.6.3. Nicotinic Receptor Agonists and Modulators

4.3.6.3.1 Acetylcholine

Acetylcholine chloride [Sigma] was dissolved in H_2O at a stock concentration of 100 mM and was applied at a concentration of 20 μM, unless otherwise stated.

4.3.6.3.2 Neostigmine

Neostigmine bromide [Sigma] is an cholinesterase inhibitor that acts to increase the amount of ACh available upon release (Burgen 1949). Neostigmine was dissolved in H_2O at a stock concentration of 20 mM and was applied at a concentration of 20-30 μM.

4.3.6.3.4 Nicotine

Nicotine [Sigma] acts as an agonist at all nAChRs with the exception of α9 homomeric, and α9/α10 heteromeric channels (Verbitsky, Rothlin et al. 2000), where it acts as an antagonist. Nicotine also causes nAChRs to desensitize much more quickly than in the presence of ACh (Chavez-Noriega, Gillespie et al. 2000). Nicotine was dissolved in 50% EtOH at a stock concentration of100 mM and applied at 5-40 μM.

4.3.6.3.3 Epibatidine

Epibatidine [Sigma] is a potent agonist at nearly all neural nAChRs. A full agonist at α2, α3, α6, and α7, epibatidine is a partial agonist at α4-containing nAChRs (Gerzanich, Peng et al. 1995; Buisson, Vallejo et al. 2000). However, like nicotine, epibatidine is also an antagonist at and α9/α10 heteromeric channels (Verbitsky, Rothlin et al. 2000). Epibatidine also causes nAChRs to desensitize much more quickly than in the presence of ACh (Buisson, Vallejo et al. 2000). Epibatidine was dissolved in H_2O at a stock concentration of 100 μM and applied at 100nM, unless otherwise stated.

4.3.6.3.4 PNU-120596

PNU-120596 [Tocris] is a positive allosteric modulator at α7 nAChRs (Hurst, Hajos et al. 2005; Gronlien, Hakerud et al. 2007). PNU-120596 was dissolved in DMSO at a stock concentration of 20 mM and applied at 1 μM. The preparation was exposed to a final concentration of 0.05% DMSO in aCSF. Control experiments were performed with only DMSO, and no physiological effects were observed at this concentration.

4.3.6.4. Drugs affecting GABAergic transmission

4.3.6.4.1 Muscimol

Muscimol [Tocris] acts as a high affinity non-selective agonist at $GABA_A$Rs (Jackson 1992). Muscimol was dissolved in H_2O to a stock concentration of 10 mM and applied at 10 μM.

4.3.6.4.2 Picrotoxin

Picrotoxin [Tocris] is an antagonist at $GABA_A$Rs. The mechanism of action of picrotoxin has been shown to be very complex. Various studies have shown evidence for the mechanism of action to be a simple open-channel block, a mixed competitive/noncompetitive inhibition, and/or non-competitive inhibition through an allosteric site to stabilize a closed or desensitized state of ligand-gated ion channels (reviewed in (Ramakrishnan and Hess 2005)). Picrotoxin was dissolved in EtOH to a stock concentration of 50 mM and applied at 25 μM.

4.3.6.4.3 Bumetanide

Bumetanide [Sigma] causes collapse of the chloride gradient of dorsal root ganglion neurons by blocking the chloride co-transporter, NKCC1 (Alvarez-Leefmans, Gamino et al. 1988; Sung, Kirby et al. 2000). Bumetanide was dissolved in EtOH to a stock concentration of 10 mM and applied at 10-50 μM.

4.3.7 Antibodies

Table 2 Antibodies for Identification of Nicotinic Subunits and Afferent Classes

Primary	*Vendor*	*Dilution*	*Secondary*	*Vendor*	*Dilution*
alpha 3	Novus Biologicals	1:500	Cy3 anti-rabbit	Jackson Immunoresearch	1:250
alpha 6	Novus Biologicals	1:500	Cy3 anti-rabbit	Jackson Immunoresearch	1:250
alpha 7	abcam	1:1000	Cy3 anti-rabbit	Jackson Immunoresearch	1:250
alpha 9	Santa Cruz Biotechnology	1:500	Biotin anti-Goat	Jackson Immunoresearch	1:250
CGRP	Serotec	1:200	Cy5 anti-Goat	Jackson	1:100

				Immunoresearch	
CGRP	Pennsylvania Labs	1:200	Cy5 anti-Guinea pig	Jackson Immunoresearch	1:100
parvalbumin	Sigma	1:1000	Cy5 anti-mouse	Jackson Immunoresearch	1:100
somatostatin	Jackson Immunoresearch	1:100	Dylight 488	Jackson Immunoresearch	1:100
CGRP	Serotec	1:200	Dylight 488anti guinea pig	Jackson Immunoresearch	1:100
CGRP	Serotec	1:200	Dylight 488anti-goat	Jackson Immunoresearch	1:100

α9 required further amplification with a tertiary label, extravidin conjugated to Cy3 at a dilution of 1:1000.

4.3.8 Determination of soma diameter

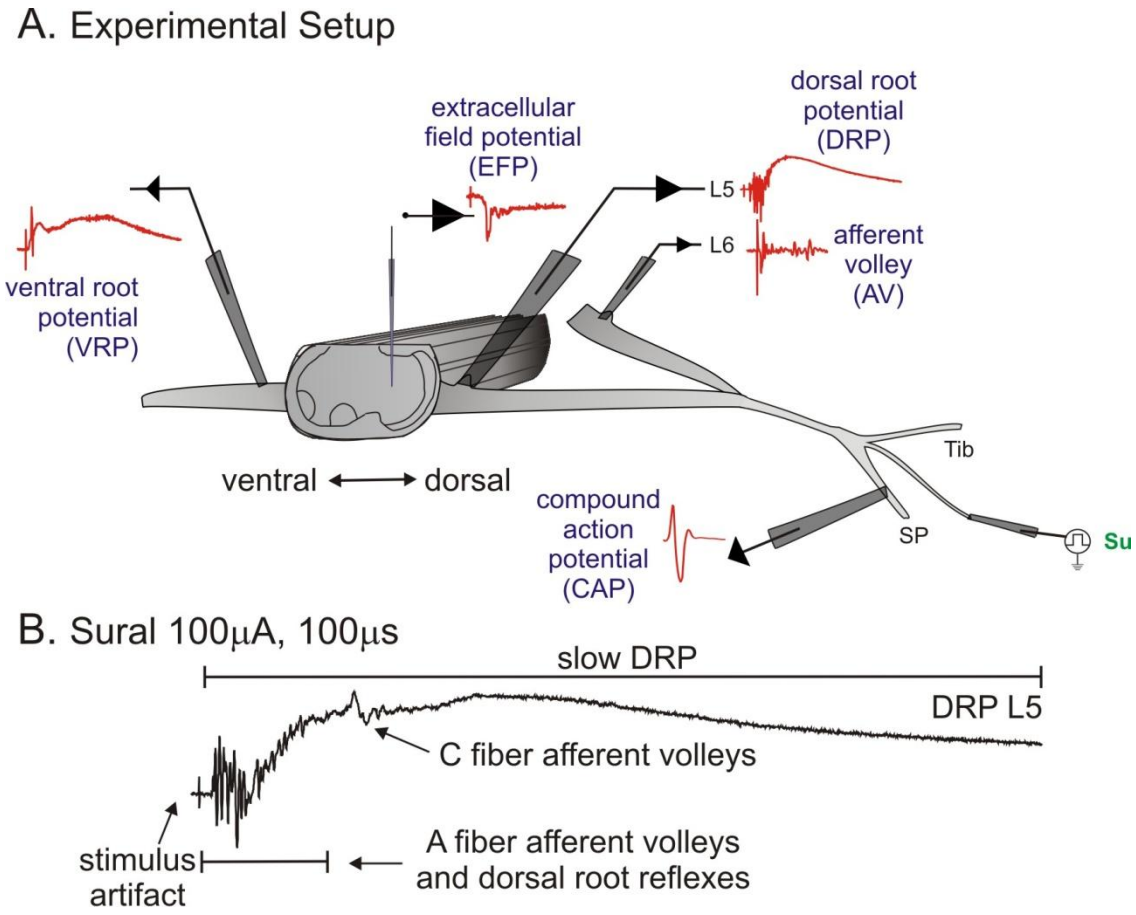
Images were captured on an Olympus Fluoview confocal microscope at 20x magnification. Next the longest and shortest diameters of the cell bodies were measured in a given frame using the Fluoview software. The diameters were averaged and the resulting value was designated as the soma diameter.

4.3.9 Data Analysis

Raw data were collected through a differential amplifier, digitized at 5-15 kHz (Digidata 1322A 16-Bit DAQ, Axon Instruments), and recorded with pClamp acquisition software (v. 10.0, Axon Instruments, Molecular Devices, U.S.A.) and stored in a PC for off-line analysis with pClamp software. DRPs were monitored using a DC-coupled amplifier, but for determination of peak amplitude, time of peak amplitude and area, each sweep was baseline subtracted to a 100 ms region immediately preceding the stimulus. The DRP was then low pass filtered at 300 Hz. Values are reported as mean ± standard deviation. Student's t-test was employed to determine statistical significance in comparing A- versus C-fiber mediated effects. When drugs were reversible, repeated measures ANOVA was used to determine significant effects by comparing to baseline control and post-washout values. When more than one drug was added before wash,

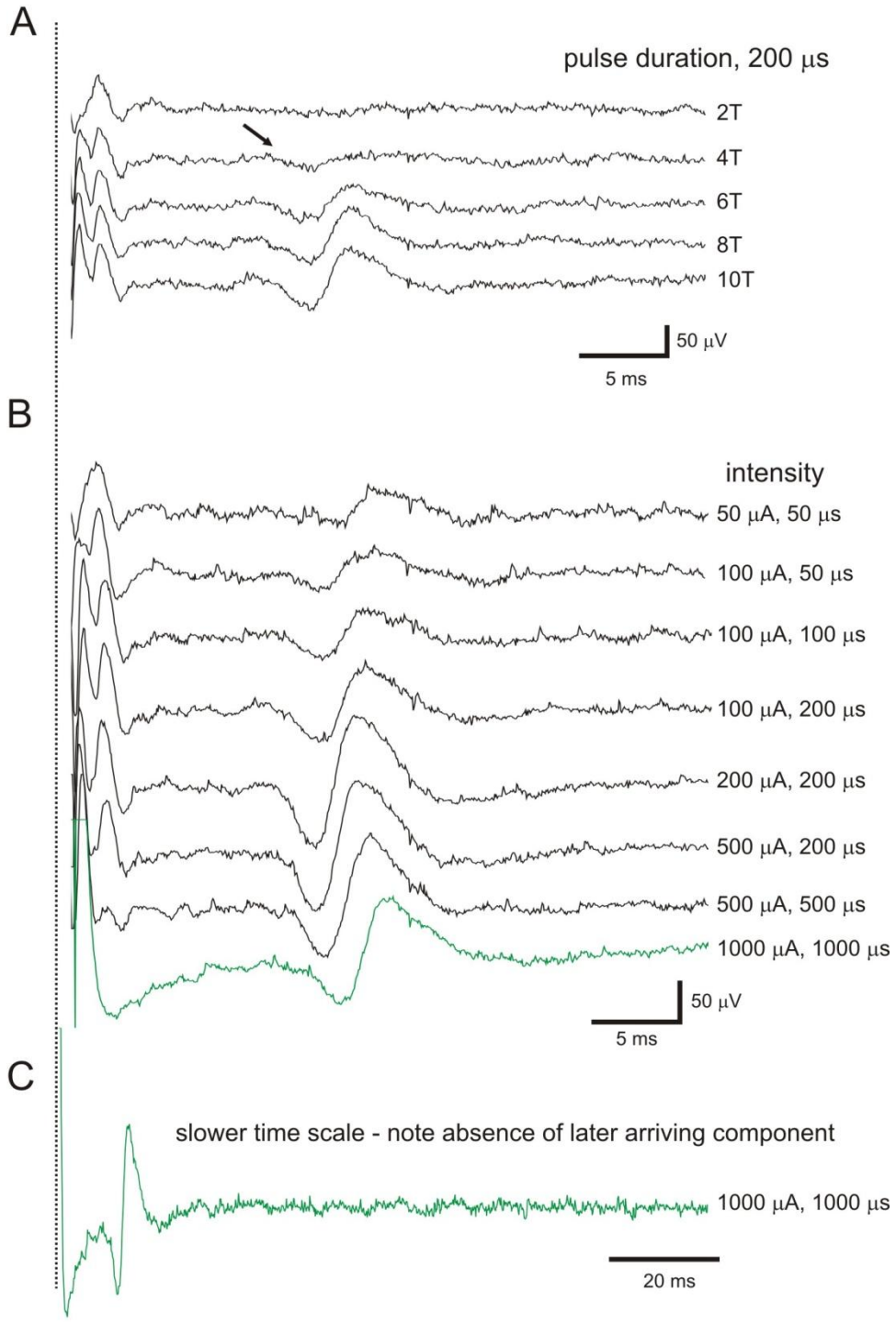
Tukey's post-hoc analysis was applied, but when the actions of only a single drug were examined, Dunnett's post-hoc analysis was performed. Unless otherwise stated, $p<0.05$ was considered as significant.

Figure 15 Experimental Design and identification of components of evoked DRPs



A. Stimulation and Recording setup with example traces. Peripheral nerves were isolated for stimulation and recording of spontaneous activity. DRPs were monitored at the dorsal root entry zone. Microelectrodes in the deep dorsal horn were used for recording of extracellular field potentials of interneurons due to afferent stimulation. **B.** The DRP shows the afferent volley from stimulated afferents followed by dorsal root reflexes atop a slow depolarizing potential. At higher stimulus intensities a C-fiber component of the DRP is produced, along with the corresponding C-fiber afferent volley.

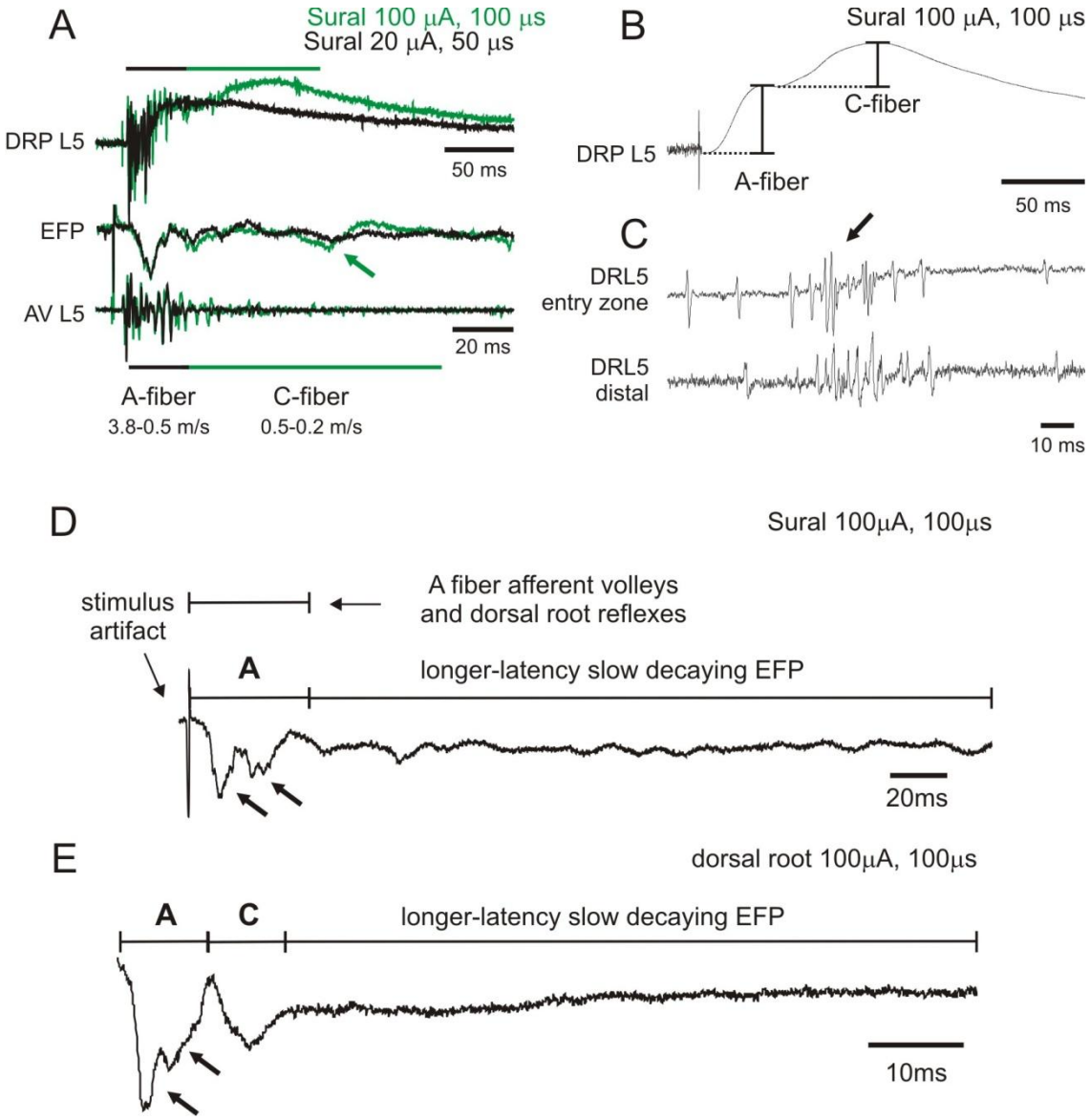
Figure 16 The effects of stimulus intensity on afferent fiber recruitment on a P9 mouse



Electrical stimuli were applied to the tibial mixed cutaneous and muscle nerve. Afferent volleys were recorded in the sciatic nerve **A.** Stimuli are delivered in multiples of threshold, and it is

clear that a later component of the afferent volley enters at 4T (arrow). **B.** Stimuli are delivered at labeled constant current intensities. The later afferent volley component is observed at constant current stimulation as low as 50 mA, 50 ms (but not at 20 mA, 50 ms - see Figure 17A). Increasing the stimulus intensity to 1000 mA, 1000 ms fails to recruit any additional components to the AV, consistent with the late component having a C-fiber identity. Interestingly, as the stimulus intensity increases, the second component of the earlier arriving A-fibers is greatly depressed. **C.** Highest stimulation intensity from panel B (in green) is displayed at a compressed time scale to show that no other slower conducting afferent volleys are recruited. In all traces, the stimulus artifact is removed for ease of viewing. Stimulation onset is identified by vertical dotted line.

Figure 17 Sample responses evoked at different stimulus intensities



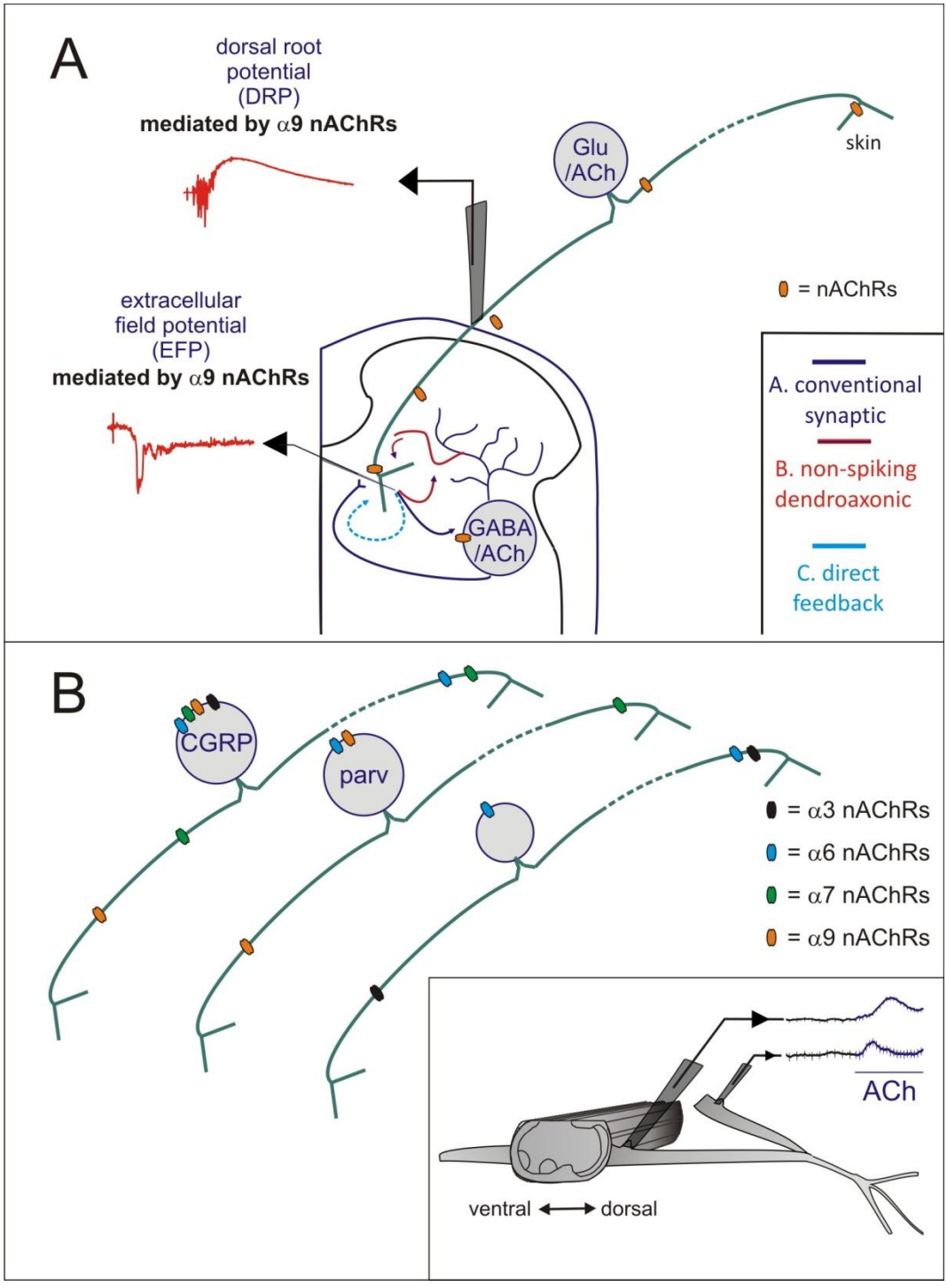
A. Cutaneous nerve stimulation, showing evoked DRP responses in a dorsal root (top), intraspinal EFPs (middle), and the recruited orthodromic afferent volley (bottom). In all signals, additional, later arriving components are observed at C-fiber stimulus strengths, as denoted by the bars above the traces. Nerves are stimulated at current intensities that recruited A-fiber (black traces) or A-

and C-fibers (green traces), as monitored by the afferent volley. C-fiber-evoked EFPs are identified by arrows. Conduction velocities for these components are listed below the bottom bars (P8 mouse). Conduction velocities were determined by measuring the delay between peripheral stimulation and arrival of the AV at the dorsal root entry zone. At the end of the experiment, peripheral nerves were dissected free and the length was measured. The values listed are the ranges for both animals used in determination of conduction velocity. **B.** Measuring the A- and C-fiber components of the DRP. The DRPs are first low pass filtered from at 100-200 Hz to remove volleys and dorsal root reflexes. Then the baseline to the inflection point of the 1st component is the amplitude of the A-fiber DRP, and from the trough of the interpeak region to the peak of the 2nd component is the C-fiber DRP amplitude. **C.** When PAD raises the membrane potential of the afferent about threshold, dorsal root reflexes are produced and propagated antidromically. These can be recorded in the dorsal root (top panel, arrow) and peripherally in the dorsal root, even when the DRP is no longer apparent. These spikes also propagate to the periphery (not shown). **D.** EFPs show 2 major components: a short latency component resulting from depolarization of interneurons monosynaptically excited by A-fiber primary afferent stimulation (labeled A) and a long latency component representing the field of the DRP, C-fiber afferent transmission, and possibly oligosynaptic transmission onto spinal neurons in the vicinity of the microelectrode. The early component of the short latency EFP generally consists of two peaks (arrows). These represent transmission from two separate populations of A-fibers. **E.** The EFP evoked by dorsal root stimulation, in addition to a short latency component mediated by A-fiber transmission, also shows another peak representing monosynaptic transmission by C-fibers, which can be analyzed separately for differential drug effects. Due to slower conduction velocity, during peripheral nerve stimulation, the C-fiber component is contaminated with the field of the DRP and possible oligosynaptic components.

4.4 Results

The following sections report the effects of ACh receptor agonists and antagonists on nerve stimulation-evoked responses on DRPs and EFPs, and DC polarization of primary afferents. I demonstrate that evoked DRPs and EFPs are mediated by α9 nAChRs. Furthermore, I show that α3, α6, α7 and α9 nAChRs are located along primary afferent axons and that activation of these receptors can lead to a direct depolarization of afferents (Figure 18). This phenomenon is independent of spinal mechanisms. While ionotropic receptors including nicotinic receptors are likely the dominant source of PAD, I briefly also explored the contribution of muscarinic receptors to the stimulus-evoked DRP as well as to DC depolarization of afferents.

Figure 18 Models for nAChR involvement in the DRP, direct polarization of afferents and nAChR distribution on primary afferents.



A. Proposed model of cholinergic contribution to the DRP and EFP. α9 nAChRs are involved in the production of both the afferent-evoked DRP and EFP. The schematic shows primary afferents projecting from the periphery into the dorsal horn of the spinal cord. Cell bodies for sensory afferents are located in the DRP. DRPs could be generated by A) excitation of cholinergic interneurons (or pathways with last-order cholinergic interneurons) which in turn synapse onto primary afferent terminals, leading to depolarization of the terminal and DRP production, B) primary afferents synapsing onto cholinergic interneurons at a dendroaxonic synapse, where the postsynaptic dendrite is depolarized and the depolarization spreads to a nearby synapse from that dendrite back onto primary afferent terminals, or C) direct activation of primary afferent terminals through a release of ACh from afferents and transmitter diffusing to extrasynaptic nAChRs. Results demonstrate nAChRs along afferent axons, as well as evidence for ACh as a transmitter released from primary afferents. Extrasynaptic nAChRs, particularly in the periphery provide additional sites of modulation of sensory signaling by nAChR activation; along the axons, themselves, or post-synaptically on spinal interneurons. **B.** The schematic summarizes immunohistochemical results for nAChR distribution on primary afferent axons and cell bodies (inset and see Figures 24-26). The activation of nAChRs on afferent axons leads to a direct depolarization of those afferents. By recording DC changes in the polarization state of afferents, I show that activation of α3, α6, α7, and α9 is able to directly depolarize primary afferents. These subunits are all expressed in varying populations of primary afferents.

4.4.1 Cholinergic transmission is involved in afferent evoked DRPs, EFPs, and DRRs

4.4.1.1 Block of muscarinic receptors does not affect cutaneous afferent evoked DRPs or EFPs

Bath applied atropine did not result in a significant change in DRP amplitude following cutaneous sural nerve stimulation. Surprisingly, following drug washout evoked DRP responses were greatly facilitated with an overall increase in area. While peak DRP amplitude of the A-fiber evoked response was unaffected, the C-fiber component of the DRP showed an increase.

The sural-evoked EFP also showed no change in the A-fiber evoked EFP peak amplitude or area. However the A-fiber EFP peak amplitude was also facilitated following drug washout (Table 3).

One possible explanation for facilitation of the DRP and EFP following washout of atropine is that block of metabotropic ACh receptors results in a compensatory increase in excitability of these circuits, seen on removal of the antagonist. Muscarinic receptors are expressed in primary afferents, (Wanke, Bianchi et al. 1994; Tata, Vilar¢ et al. 1999), but the lack of effect of atropine on drug addition suggests that muscarinic receptors have little direct contribution to the DRP and EFP production.

Table 3 Atropine has little effect on the DRP and EFP evoked by sural nerve stimulation

A-fiber DRP		**Peak Amplitude (% of control)**	
10 μM atropine	n=4	80 +/- 14	
wash		161 +/- 87	
C-fiber DRP			
10 μM atropine	n=4	121+/- 33	
wash		*200 +/- 20***	
EFP		**Peak Amplitude (% of control)**	**Area (% of control)**
10 μM atropine	n=3	101 +/- 20	99 +/- 14
wash		*159 +/- 115**	143 +/- 73

*p<0.05; **p<0.01 All values are expressed as percent of control. Control values were measured immediately before drug application. DRPs and EFP were evoked by stimulation of the sural nerve at 100 μA, 100 μs. Application of atropine has no effect on the DRP or EFP. However, there is an increase in the C-fiber DRP and EFP amplitude following washout.

4.4.1.2 The α9 nAChR contributes to DRPs and EFPs evoked by afferent stimulation

To examine the contribution of nicotinic receptors on afferent evoked DRPs, DRRs, and EFPs, I applied a series of nAChR agonists and antagonists. For several experiments, peripheral nerves were dissected out and stimulated to examine drug actions in a solely cutaneous afferent population. Peripheral nerve stimulation also allowed marked separation of A- and C-fiber evoked components of the DRP. I found that d-tubocurarine, α-bungarotoxin and mecamylamine reduced the DRP evoked DRRs. These drugs have overlapping actions at the α9 nicotinic receptor. d-tubocurarine and α-bungarotoxin also depressed the EFP, particularly the slower conducting A-fiber component. In contrast, application of DHβE, which is an antagonist at α2, α3, and α4-containing nAChRs, but not α9, had no effect on the DRP or DRRs. Also, MLA, the α7 nAChR antagonist had no effect on the DRP or DRRs. These findings are described in detail below.

4.4.1.2.1 d-tubocurarine, α-bungarotoxin and mecamylamine depress the DRP

Applied at 10-20 μM, d-tubocurarine depressed both the amplitude and overall area of the dorsal root stimulus-evoked DRP while time to peak of the DRP was unchanged (Table 4; Figure 19A; n=6). These actions were largely irreversible following drug washout. As the AV was unaffected by d-tubocurarine (n=3/4; 20 μM; not shown) observed effects are via actions independent of afferent spike generation/propagation.

To determine whether depressant actions were selective to subpopulations of evoked afferent fibers, I separated A from C fiber evoked components of the DRP based on their separate times to peaks following dorsal root stimulation (n=3). Both A- and C-fiber components underwent a similar reduction (Table 5). Likewise, following stimulation of cutaneous afferents, both A- and C-fiber components of the DRP were similarly reduced (Figure 19A).

In order to determine efficacy of d-tubocurarine's action and potential concentration-dependent differences in the A- and C-fiber populations, I examined dose-response relations following cutaneous afferent stimulation. d-tubocurarine depressed both A- and C-fiber DRP components in a dose-dependent manner, and no difference was seen in the resulting curves. IC_{50} values for A- and C-fibers were comparable at 5.18 μM and 7.45 μM, respectively (Figure 19B).

α-bungarotoxin also decreased the amplitude and area of the DRP resulting from dorsal root stimulation with no effect on time to peak (n=9 [7 rat, 2 mouse]; Figure 19C; Table 4). α-bungarotoxin's actions were only partially reversible upon washout, as it is an irreversible agonist at all nAChRs with the exception of α9. There was no change in the AV on drug addition (data not shown).

While the effect was not as pronounced as with d-tubocurarine, and α-bungarotoxin, mecamylamine (30-50 μM) reduced DRP area to 90±8% of control DRP area (Table 4) This decrease in area is partly attributable to a shortened DRP duration (Figure 19D). However, there was no effect on DRP amplitude or time to peak (Table 4). A- and C-fiber components of the DRP were differentiable in 2 experiments, one with sural nerve stimulation and the other with dorsal root stimulation. In these experiments, the C-fiber component appeared preferentially depressed by mecamylamine (Table 5), but the small sample size precluded statistical analysis of significance.

Six experiments were conducted in the presence of MLA, a specific α7 nAChR antagonist. Four involved dorsal root stimulation (2 rats, 2 mice 0.2-1.0 μM), while the other two involved stimulation of peripheral nerves (all at 100μA, 100 μs). MLA had no effect on time to peak, DRP amplitude or DRP area (Table 4). When A- and C-fiber components of the DRP were separable, there was again no change after drug application (n=3) (two with peripheral nerve stimulation, one with dorsal root stimulation). The AV was monitored in 3 experiments, and there was no

obvious effect on drug application (data not shown). Likewise, DHβE did not affect the DRP (Table 4).

A more robust depression of the DRP was observed in the presence of d-tubocurarine and α-bungarotoxin when compared to mecamylamine. One possibility for the discrepancy in these drugs actions could be the application times. In 2 of the experiments with mecamylamine, the drug was only applied for 5 minutes, so drug effects may be underestimated.

Table 4 d-tubocurarine, α-bungarotoxin and mecamylamine depress the DRP

DRP		**Peak Amplitude (% of control)**	**Time to Peak (% of control)**	**DRP Area (% of control)**
10-20 µM d-tubocurarine	n=6	*58 +/- 18***	104 +/- 5	*60 +/- 16***
Wash	n=5	*71 +/- 26**	101 +/- 3	*67 +/- 28**
100 nM α-bungarotoxin	n=9; 7 rat, 2 mouse	*69 +/- 13**	99 +/- 3	*62 +/- 14**
Wash	n=6; 4 rat, 2 mouse	77 +/- 46	101 +/- 6	77 +/- 45
30-50 µM mecamylamine	n=7	93 +/- 8	100 +/- 1	*90 +/- 8**
Wash	n=3	*119 +/- 22**	99 +/- 1	*113 +/- 26**
200 nM – 1 µM MLA	n=6	95 +/- 27	100 +/- 2	90 +/- 25
Wash	n=3	*66 +/- 8**	103 +/- 5	*52 +/- 27**
50 µM DHβE	n=3; 1 rat, 2 mice	95 +/- 4	100 +/- 1	90 +/- 14

*p<0.05; **p<0.01. DRPs were elicited by dorsal root or sural nerve stimulation at 100 µA, 100 µs .d-tubocurarine and α-bungarotoxin depress DRP amplitude. Mecamylamine depresses the area of the DRP alone. MLA and DHβE have no effect on the DRP. However, MLA shows a depression of the DRP amplitude and area on washout. None of the drugs examined affected the time to peak of the DRP. In several experiments, washout effects could not be determined due to subsequent application of another drug that affected the DRP.

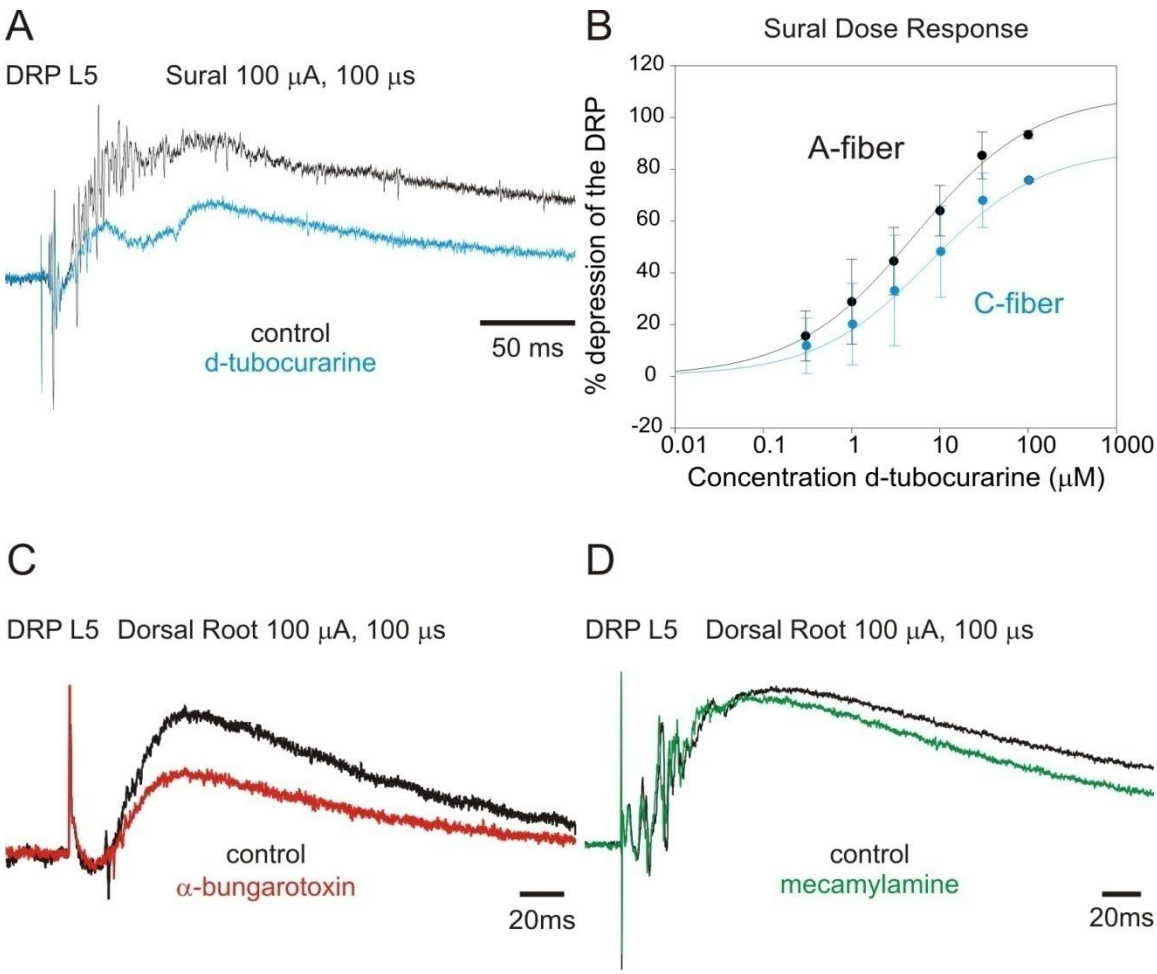
Table 5 A- and C-fiber components of the DRP are depressed by nAChR antagonists

A-fiber-evoked DRP amplitude		**Peak Amplitude (% of control)**
20 µM d-tubocurarine	n=3	*55 +/- 29***
Wash	n=3	*70 +/- 34**
30-50 µM mecamylamine	n=3	86 +/- 10
200 nM MLA	n=3	101 +/- 9
C-fiber evoked DRP amplitude		
20 µM d-tubocurarine	n=3	*54 +/- 20***
Wash	n=3	*49 +/- 22**
30-50 µM mecamylamine	n=3	52 +/- 30
200 nM MLA	n=3	98 +/- 21

*p<0.05; **p<0.01DRPs were evoked by dorsal root or sural nerve stimulation at 100 µA, 100 µs.

d-tubocurarine similarly depresses both the A- and C-fiber components of the DRP. Mecamylamine shows a trend towards depression of the C-fiber component of the DRP. MLA does not affect the A- or the C-fiber component of the DRP. Washout was not examined for mecamylamine or MLA.

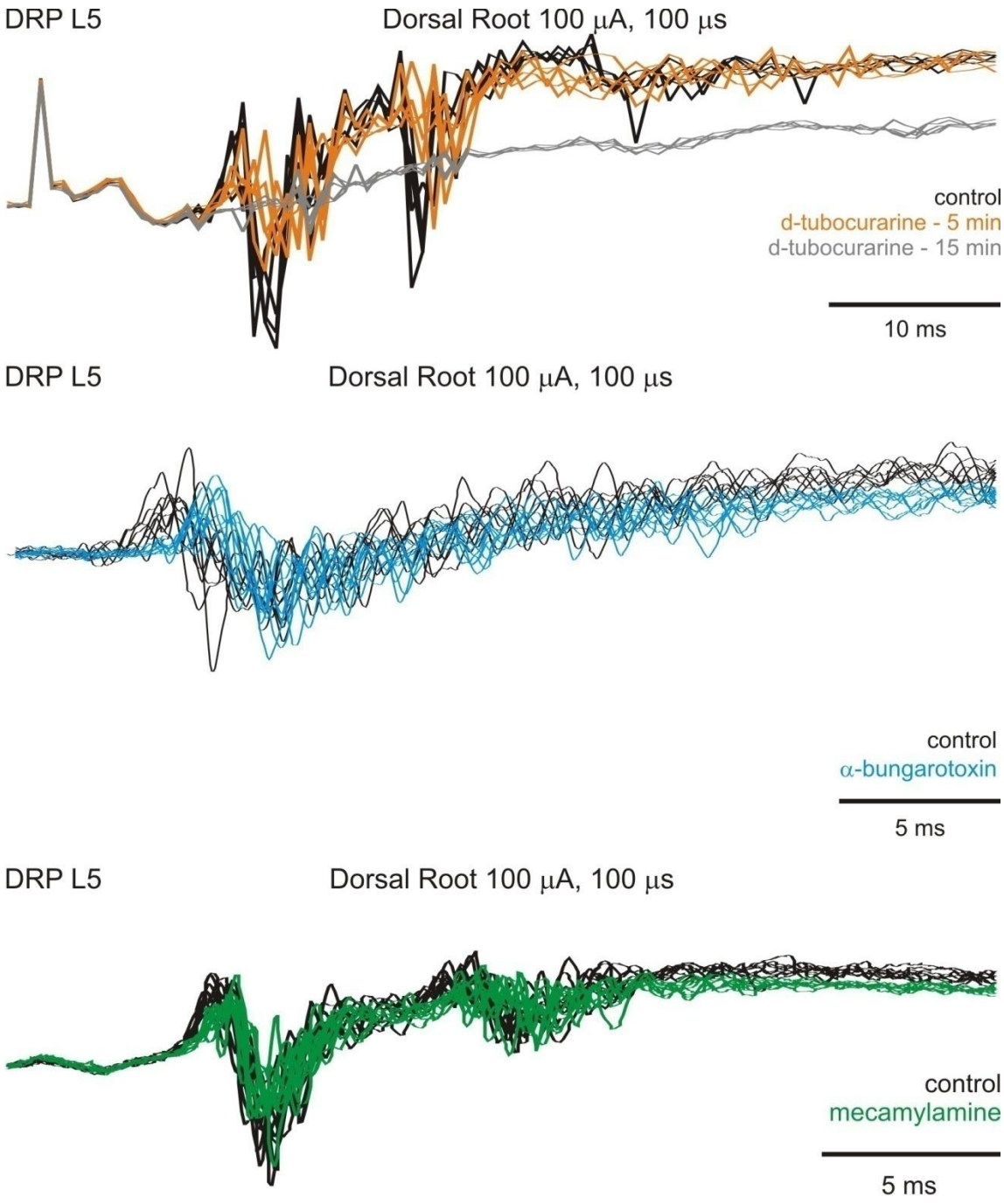
Figure 19 Effects of nicotinic antagonists on evoked DRPs



A. d-tubocurarine (10 μM) depresses the DRPs (top row) and EFPs (bottom row) in cutaneous (sural; 100 μA, 100 μs). Both A- and C-fiber components of the DRPs are depressed. **B.** d-tubocurarine dose-response relations for depression of the DRP following sural nerve stimulation. Curves are separated into A- and C-fiber components. Note the greater depression of the A-fiber component. **C.** L4 dorsal root stimulation at 100μA, 100 μs. The DRP is depressed by 100nM a-bungarotoxin. **D.** Stimulation of the dorsal root at 100 μA, 100 μs. Mecamylamine (50 μM) decreases the area of the DRP seen as a decrease in DRP duration. The lack of effect on the peak amplitude of the DRP suggests a preferential reduction in A-fiber dorsal root reflexes and the C-fiber DRP. There is a facilitation of DRP amplitude after drug washout (not shown).

I also examined nAChR contributions to the production of evoked dorsal root reflexes (DRRs). In all cases where I observed dorsal root reflexes (n=10), d-tubocurarine at 20 μM greatly depressed or abolished DRRs following stimulation of the dorsal root (n=6/6) and peripheral nerves (cutaneous n=2/2). I observed a distinct difference in the onset of depression of DRPs and DRRs (Figure 20A). It is clear the DRR depression occurred before the DRP was affected, suggesting that DRP and DRRs are produced by independent events. Another interpretation is that the afferents that are undergoing PAD to produce a DRP are not the same afferents that DRRs are being produced in. This could only be resolved through intraaxonal recordings from single afferents to determine if the same afferents involved in the DRP also showed DRRs. α-bungarotoxin also was able to depress DRRs (Figure 20B). However, in comparison to d-tubocurarine, the block was coincident with depression of the DRP (n=6).While mecamylamine did not depress the early component of the DRP; DRRs on the rising phase of the DRP were depressed in all five experiments where DRRs were observed (Figure 20C). DRRs were not affected by MLA (n=6) or DHβE (n=4) (data not shown).

Figure 20 Effects of nAChR antagonists on dorsal root reflexes



To show DRRs, five-ten overlapping raw data traces for both control and drug conditions are shown. **A.** Application of d-tubocurarine (20 μM) leads to a decrease in the dorsal root reflex (DRR) seen superimposed on the DRP (time regions denoted by black bar). The DRP was evoked

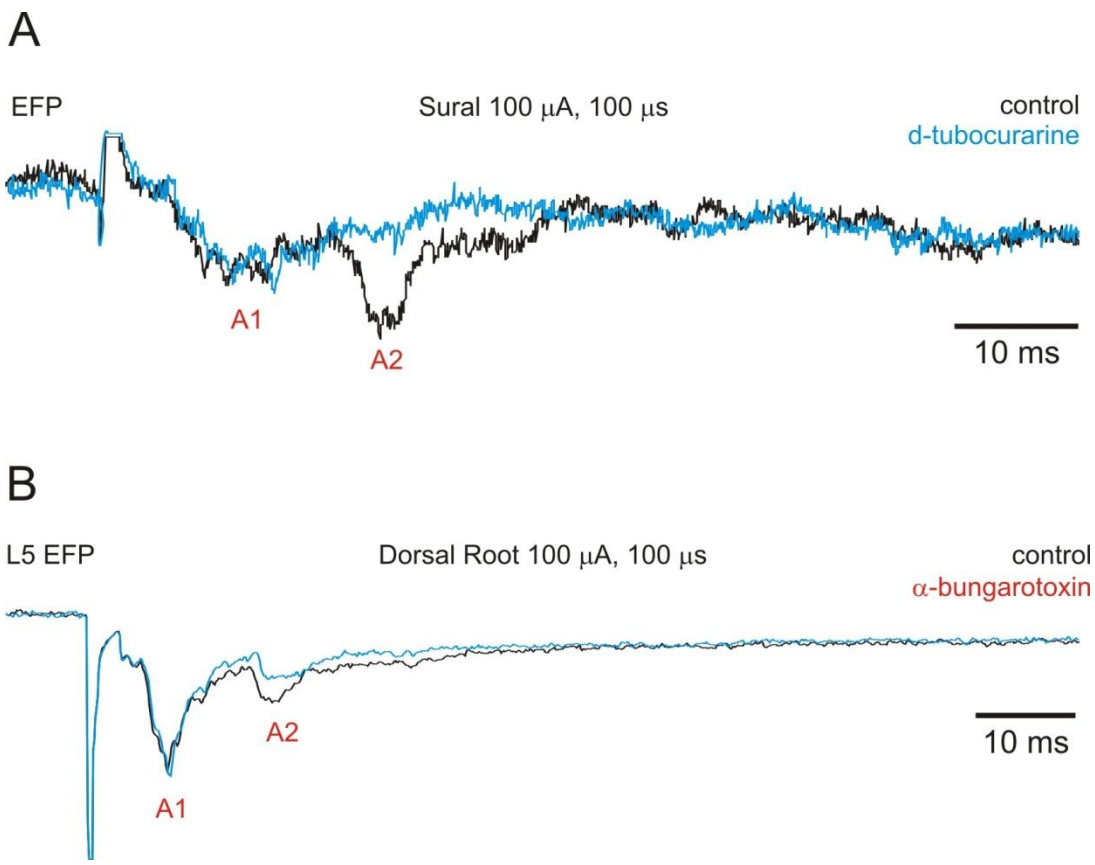
following dorsal root stimulation (c) and raw superimposed traces are shown in control and at two time points after d-tubocurarine application. Note that the DRR depresses prior to the DRP amplitude (orange traces). This is followed in time by depression of the DRP (gray traces). **B.** Dorsal root stimulation at 100 µA, 100 µs. a-bungarotoxin leads to a decrease in the DRR concurrent with the decrease in the DRP. **C.** Mecamylamine (50 µM) decreases the dorsal root reflex (time denoted by the black bar) evoked by dorsal root stimulation (100 µA, 100 µs) before any depression is seen in the DRP amplitude, suggesting an independent mechanism of action on DRRs and DRPs.

Intraspinal extracellular field potentials (EFPs) reflect population post-synaptic potential in spinal interneurons resulting from afferent transmission. EFPs were examined in 7 experiments: 5 with peripheral nerve stimulation, and 2 with dorsal root stimulation. There were no obvious differences in actions arising from A-fibers in stimulation of peripheral nerves or dorsal roots, so all experiments were combined for analysis. d-tubocurarine significantly depressed both initial slope (61±32% of control) and overall area (54±34% of control) of the A-fiber-evoked EFP with no change in time to peak (n=7). As with DRPs, observed effects did not recover on washout.

Commonly, A-fiber evoked EFPs had two separable peaks. d-tubocurarine preferentially depressed the second EFP, corresponding to transmission from slower conducting A-fibers, with the first peak being largely unchanged (Figure 21A). Quantification of the amplitudes of these two separate peaks reveals that, d-tubocurarine preferentially depresses the second peak when compared to the first peak (37±12% and 93±27% of control, respectively; $p<0.01$, n=5). When the C-fiber component of the EFP could be measured following dorsal root stimulation (n=2), there was also a trend toward depression of the EFP amplitude to 44±39% of control (data not shown). Significance could not be determined due to the small sample size.

EFPs in the presence of α-bungarotoxin were monitored in two experiments following dorsal root stimulation. There was a decrease in the overall area of the A-fiber field (82±4% of control; $p < 0.05$) (one mouse, one rat, 100 nM). The A-fiber mediated field could be separated into two components (n=2), and there was a trend toward preferential depression of the second peak (93±17% versus 74±5% of control) (Figure 21B). This effect was not considered significant due to the small sample size.

Figure 21 d-tubocurarine and a-bungarotoxin preferentially depress the second component of the A-fiber mediated EFP



EFPs were recorded in the deep dorsal horn where stimulation of the sural nerve or dorsal root elicited the largest response. **A.** d-tubocurarine preferentially depressed the second component of the A-fiber EFP (A2). **B.** The dorsal root was stimulated at 4T. While a-bungarotoxin does not significantly affect the A1 component of the EFP, there is a preferential depression of the second component of the A-fiber field (A2).

The nicotinic receptor antagonists, α-bungarotoxin, d-tubocurarine, and mecamylamine depressed the DRP and DRRs. These drugs have overlapping actions at the α9 nAChR. When separable, the A- and C-fiber components were depressed by d-tubocurarine. EFPs were depressed also by α-bungarotoxin and d-tubocurarine, which have overlapping actions at α7 and α9 nAChRs. Overall these results demonstrated that the α9 nAChR is involved production of the DRP, generation of DRRs, and transmission from slower conducting A-fibers, as evidenced by a preferential reduction in the second component of the EFP. As both the EFP and DRP are depressed by nicotinic antagonists, it is possible that least part of the depression of the A-fiber-evoked DRP is due to inhibition of monosynaptic transmission from the slowly conducting A-fibers involved in production of the DRP.

4.4.1.3 ACh and nicotinic receptor agonists depress DRPs and EFPs likely through depolarization block of afferent signaling.

ACh, when added to the bath, significantly decreased DRP amplitude and area (Figure 22A; Table 6). These effects were reversible on washout. These experiments were performed with dorsal root stimulation, where it was not possible to differentiate between the A- and C-fiber components of the DRPs.

The cholinesterase inhibitor neostigmine acts by increasing the amount of ACh available upon release (Burgen 1949). In thirteen experiments, when stimulating the dorsal root, the A- and C-fiber components of the DRP could not be separated for analysis. The DRP amplitude and area is depressed following neostigmine (Table 6). Subsequent application of ACh led to a further depression of DRP amplitude and area. The depression seen after subsequent application of ACh is also significantly different than after neostigmine alone ($p<0.01$). These effects were partially reversible on washout (Table 6).

ACh/neostigmine also depressed the peak DRP amplitude of the A-fiber component of the DRP (n=3; Figure 22B; Table 7) following sural nerve stimulation. These effects were largely

reversible. While the C-fiber component is numerically depressed after ACh/neostigmine to 42±28% of control amplitude, the change was not considered significant.

As the effects of ACh and neostigmine added exogenously could involve both muscarinic and nicotinic components, and the focus of the study was to determine nicotinic receptor contributions to sensory processing, the actions of the specific nicotinic receptor agonists, epibatidine and nicotine, were studied. As the following agonists tested have different affinities for various subunit-containing nAChRs, they also provide some insight into the specific nAChRs involved in observed actions on primary afferents.

Application of nicotine produced a reversible depression of the DRP amplitude and area (n=4; Figure 22C; Table 6). These effects were partly reversible. Nicotine acts as an agonist at all nAChRs with the exception of α9 homomeric, and α9/α10 heteromeric channels, where it is an antagonist (Verbitsky, Rothlin et al. 2000). Thus, it cannot be determined if the depression of the DRP is due to depolarization block of afferent signaling block or through antagonism of the α9 or α10 nAChRs involved in the production of the DRP.

Epibatidine is a potent agonist at nearly all neural nAChRs including as a full agonist at α2, α3, α6, and α7, and partial agonist at α4-containing nAChRs (Gerzanich, Peng et al. 1995; Buisson, Vallejo et al. 2000; Alexander, Mathie et al. 2007). Like nicotine, epibatidine is also an antagonist at and α9/α10 heteromeric channels (Verbitsky, Rothlin et al. 2000).In 5 experiments, either cutaneous (2), or dorsal roots (3) were stimulated at 4T, and C-fibers were not recruited, so only a single component of the DRP could be analyzed. There did not appear to be a difference in drug actions on cutaneous versus mixed afferent populations, so they were combined for analysis. Epibatidine reversibly depressed DRP amplitude (n=5; Figure 22D) with a non-significant numerical reduction in mean DRP area (p=0.20). In 2 experiments with afferent stimulation at 10T (sural or dorsal root), A- and C-fiber components of the DRP appeared

similarly depressed (Table 7) suggesting common actions of epibatidine on these two classes of afferents. However, due to the small sample size, these changes were not considered significant.

PNU-120596 (PNU) is part of a class of positive allosteric modulators that specifically targets the α7 homomeric nAChR (Hurst, Hajos et al. 2005; Gronlien, Hakerud et al. 2007). PNU evokes currents, even when the α7 receptor is otherwise completely desensitized, suggesting that it stabilizes a desensitized-open state that differs from the regular open state stabilized by agonists (Bertrand and Gopalakrishnan 2007). Stimulation of the sural nerve at 100 μA, 100 μs evoked a DRP with separable A- and C-fiber components. PNU did not have an effect on either component of the DRP (Table 7; n=3).

The depression of the DRP seen with application of ACh of nAChR agonists is likely due to block of afferent signaling by nAChR activation on primary afferents. Also, with afferent depolarization, the membrane potential of primary afferents will approach the reversal potential of the DRP leading to an apparent decrease in DRP amplitude.

Table 6 ACh and nicotinic agonists depress the DRP

DRP		**Peak Amplitude (% of control)**	**Time to Peak (% of control)**	**Area (% of control)**
20-50 µM acetylcholine	n=10	74 +/- 22*	100 +/- 1	66 +/- 25*
wash		92 +/- 16	100 +/- 0	95 +/- 19
20-30 µM neostigmine	n=13	70 +/- 12*	100 +/- 2	64 +/- 13*
20-30 µM acetylcholine	n=13	31 +/- 11**	99 +/- 2	20 +/- 9**
wash	n=13	72 +/- 46	100 +/- 3	67 +/- 43
5-40 µM nicotine	n=4	72 +/- 20*	100 +/- 1	61 +/- 31*
wash	n=4	79 +/- 10	100 +/- 1	83 +/- 6
100nM epibatidine	n=5	77 +/- 17*	99 +/- 1	61 +/- 38
wash	n=5	84 +/- 12	100 +/- 1	110 +/- 64

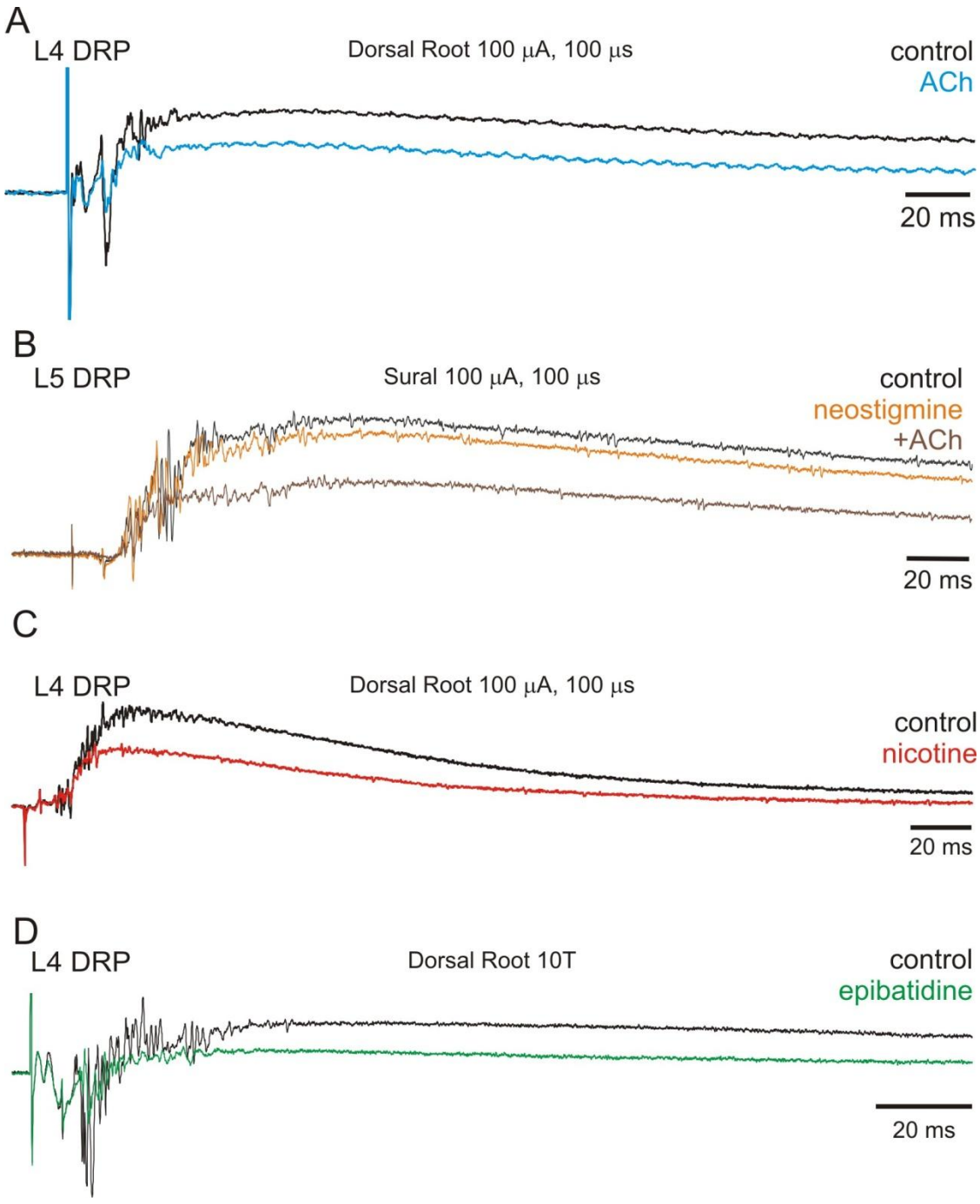
*p<0.05; **p<0.01. DRPs were elicited by dorsal root stimulation of 100 µA, 100 µs for ACh, neostigmine + ACh, and nicotine, and 4T for epibatidine. ACh alone depresses the DRP peak amplitude and area, as do neostigmine in combination with ACh. Nicotine also depresses the DRP amplitude and area. Epibatidine depresses the peak amplitude.

Table 7 Effects of cholinergic agonists on the A- and C-fiber components of the DRP

A-fiber-evoked DRP		**Peak Amplitude**
20 µM neostigmine	n=3	97 +/- 7
20 µM ACh	n=3	54 +/- 11*
wash	n=3	84 +/- 13
100nM epibatidine	n=2	62 +/- 10
wash	n=2	75 +/- 13
1µM PNU-120596	n=3	101 +/- 4
wash	n=3	72 +/- 23
C-fiber-evoked DRP		
20 µM neostigmine	n=3	84 +/- 8
20 µM ACh	n=3	42 +/- 28 (p=0.20)
wash	n=3	84 +/- 50
100nM epibatidine	n=2	56 +/- 14
wash	n=2	80 +/- 12
1µM PNU-120596	n=3	105 +/- 11
wash	n=3	95 +/- 36

*$p<0.05$. DRPs were evoked by dorsal root stimulation at 10T or sural nerve stimulation at 100 µA, 100 µs. ACh and neostigmine together depressed the A-fiber DRP and there was a trend toward depression of the C-fiber DRP. Epibatidine appeared to depress both the A- and C-fiber components of the DRP, but significance could not be determined due to a small sample size. The α7 positive allosteric modulator PNU-120596 had no effect of the A- or C-fiber DRP amplitude.

Figure 22 ACh and nicotinic agonists depressed the DRP



In general drugs were bath applied for 5 minutes. Dorsal roots or peripheral nerves were stimulated every 15 seconds. The final four episodes of stimulation were averaged. **A.** The dorsal root was stimulated at 100 μA, 100 μs. ACh (50 μM) reversibly depressed the DRP

following dorsal root stimulation. **B.** Sural nerve stimulation at 100 µA, 100 µs: ACh (20 µM) following neostigmine (20 µM) reversibly depressed the A-fiber component of the cutaneous afferent evoked DRP. **C.** Stimulating the dorsal root at 100 µA, 100 µs, nicotine (40 µM) depressed DRP amplitude and area. **D.** Stimulation of the dorsal root at 10T: Epibatidine (100 nM) depressed both the A- and C-fiber components of the DRP.

EFPs resulting from dorsal root stimulation at 100 μA, 100 μs were monitored in four experiments where ACh was applied. In all four the early peak of the EFP was separated into two components representing monosynaptic transmission onto interneurons from two distinct populations of A-fibers. A C-fiber component was also clearly discernible during dorsal root stimulation (Figure 23Ai, labeled A1, A2 and C). The first A-fiber mediated field is not significantly affected by ACh. However, the second A-fiber mediated field is depressed to 56±31% of control ($p=0.03$). The C-fiber field is also depressed to 62±21% of control ($p=0.05$). ACh did not significantly alter the area, or slope of the early A-fiber EFP component (79±22, and 69±22% of control values respectively; Figure 23Ai).

In four experiments the antidromic volley (AV) was recorded at a site on the dorsal root distal to the stimulating electrode (2 at 100 μA, 100 μs; 2 at 10T). While ACh (50 μM) had no apparent effect on the A-fiber component of the AV, in all four cases the C-fiber component of the AV depressed and slow with drug application(Figure 23Aii). The effects of ACh on the DRP, EFP and AV are likely a result of depolarization of primary afferents (see Figure 24B for ACh-induced DC depolarization).

I examined the effects neostigmine alone and in combination with ACh on the EFP produced by cutaneous afferent and dorsal root stimulation. The early (A-fiber) component of the cutaneous afferent evoked EFP was separated into two components (A1 and A2). Neither component was affected by neostigmine (A1 = 98±35%; A2 = 79±29%). However, subsequent addition of ACh ($n=5$) decreased the first peak (A1) to 47±36% of control amplitude ($p<0.05$ compared to control and $p<0.05$ compared to neostigmine). The second peak (A2) was similarly depressed in the presence of ACh to 54±27% of control ($p<0.05$). Application of ACh also depressed the initial slope (42±35% of control slope) of the EFP, though the area was not significantly altered (49±45% of control; Figure 23, top left panel). All effects were partially reversible after wash

(A1 and A2 amplitudes to 78±18% and 68±31%, slope to 64±29% and area to 66±34% of control values (data not shown).

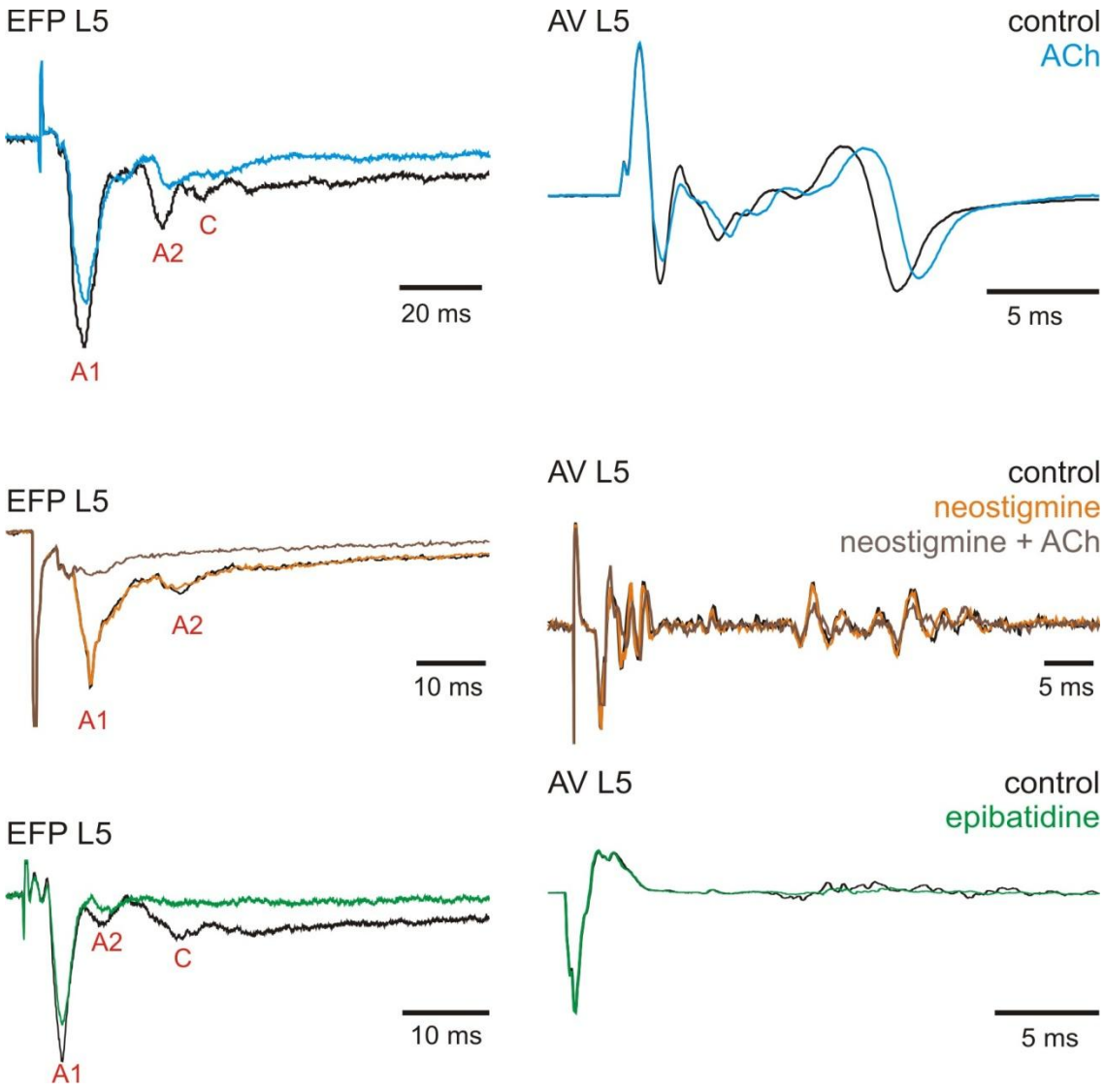
Stimulation of the dorsal root did not produce an A-fiber field that could be separated into multiple components, but a C-fiber component was clearly distinguishable (n=4; data not shown). Upon application of neostigmine, the A- and C-fiber components showed a numerical, but not significant decrease to 79±26% and 49±26% of control, respectively. Subsequent application of ACh, however, led to a significant depression of both components. The depression of the C-fiber component was significantly greater than depression of the A-fiber component. The A-fiber component depressed to 36±12% of control ($p<0.001$). The C-fiber component depressed to 16±10% of control ($p<0.01$). On washout, the A-fiber component showed complete reversibility, while the C-fiber component only partially recovered (93±16% compared to 59±14; $p<0.01$). This suggests that ACh may have longer lasting effects on C-fiber afferent transmission, possibly due to comparatively longer repolarization latency in C fibers (data not shown).

Neostigmine did not affect the AV in six experiments where it was monitored, neither during dorsal root stimulation (n=3; 100 µA, 100 µs), nor during cutaneous nerve stimulation (n=3; 100 µA, 100 µs) (Figure 23, middle right panel). Subsequent addition of ACh, however, led to a decrease in the C-fiber AV following cutaneous nerve stimulation (n=3; Figure 23, middle right panel).

Similarly, while a small sample size (n=2) did not allow for determination of significance, epibatidine appeared to decrease EFP amplitude, area, and initial slope (Figure 23Ci). In these experiments, the A-fiber component of the EFP was separated into two components, and there was a trend toward a preferential decrease in the second component of the A-fiber mediated EFP (64±18% versus 32±14% of control).When monitored, epibatidine had no effect on the AV (n=2, Figure 23Cii).

ACh and nicotinic agonists depress afferent evoked signaling from primary afferents; both the DRP and EFP are depressed. There do not appear to be preferential effects on a particular afferent subtype. The depression of these signals is likely due to direct afferent depolarization through activation of extrasynaptic nAChRs along primary afferent axons, which is discussed in detail below. However, I cannot rule out the possibility of depolarization block of interneuronal activity in addition to affects directly on primary afferents.

Figure 23 Ach, Ach + neostigmine and epibatidine depress EFPs



EFPs were recorded from the deep dorsal horn in an area where stimulation of the dorsal root or a cutaneous nerve at 100 µA, 100µs or 10T elicited the greatest response. In general drugs were applied for five minutes and afferents were stimulated every 15 seconds. The final four episodes were averaged. **Ai&Aii**. All components of the EFP evoked by 100 µA, 100µs stimulation of the dorsal root appear depressed by ACh. Overall there is a significant depression of the late A-fiber component of the EFP (A2), as well as a depression of the C-fiber component (C). The earliest A-fiber mediated field is not significantly depressed (A1). The afferent volley appeared to decrease

slightly and slowly, particularly the later components. **Bi&Bii.** SP stimulation at 4T: Both A-fiber mediated components of the EFP (A1 and A2) are unaffected by neostigmine, but depressed following subsequent addition of ACh. The C-fiber component of the AV is depressed following ACh. **Ci&Cii.** While there is no significant effect of epibatidine on the EFP as a population, in this example there is an observable decrease in all components, with a near complete block of the C-fiber mediated EFP (C). The AV is unchanged in the presence of epibatidine.

4.4.2 Application of ACh and nicotinic agonists leads to a depolarization of primary afferents

Cholinergic receptor contributions to primary afferent membrane potential were measured by recording the polarization state of primary afferent by means of a DC coupled suction electrode attached *en passant* to a lumbar dorsal root (L3-L5). DC recordings provide a measure of changes in the membrane potential for the population of primary afferents sampled, dubbed DC depolarizations. I first examined cholinergic depolarization of primary afferents with possible contributions from spinal circuitry. Then I undertook experiments to demonstrate that much of the cholinergic depolarization of afferents is direct, resulting from activation of nAChRs on primary afferents themselves.

In order to determine whether AChR antagonists altered ACh and AChR agonist-induced DC depolarizations, control experiments were first employed to determine reproducibility of responses to repeated agonist exposure. Table 8 shows the effects of repeated applications of ACh to the magnitude of observed DC polarization. While the 2nd application of ACh did not show a significant change of the DC depolarization in comparison to the first application; on the 3rd application, DC depolarization is significantly depressed in amplitude from the first application ($p<0.05$). As there is a depression with repeated applications of ACh, antagonist applications in the presence of agonist were commonly bracketed by agonist-alone applications in order to ensure that any changes in the level of ACh-induced DC depolarizations were a result of drug action, not merely repeated application.

Table 8 Repeated applications of ACh leads to a decrease in depolarization magnitude

20 µM ACh Application		**Depolarization(% of control ACh depolarization)**
2nd application	n=4	67 +/- 37
3rd application	n=4	41 +/- 27*

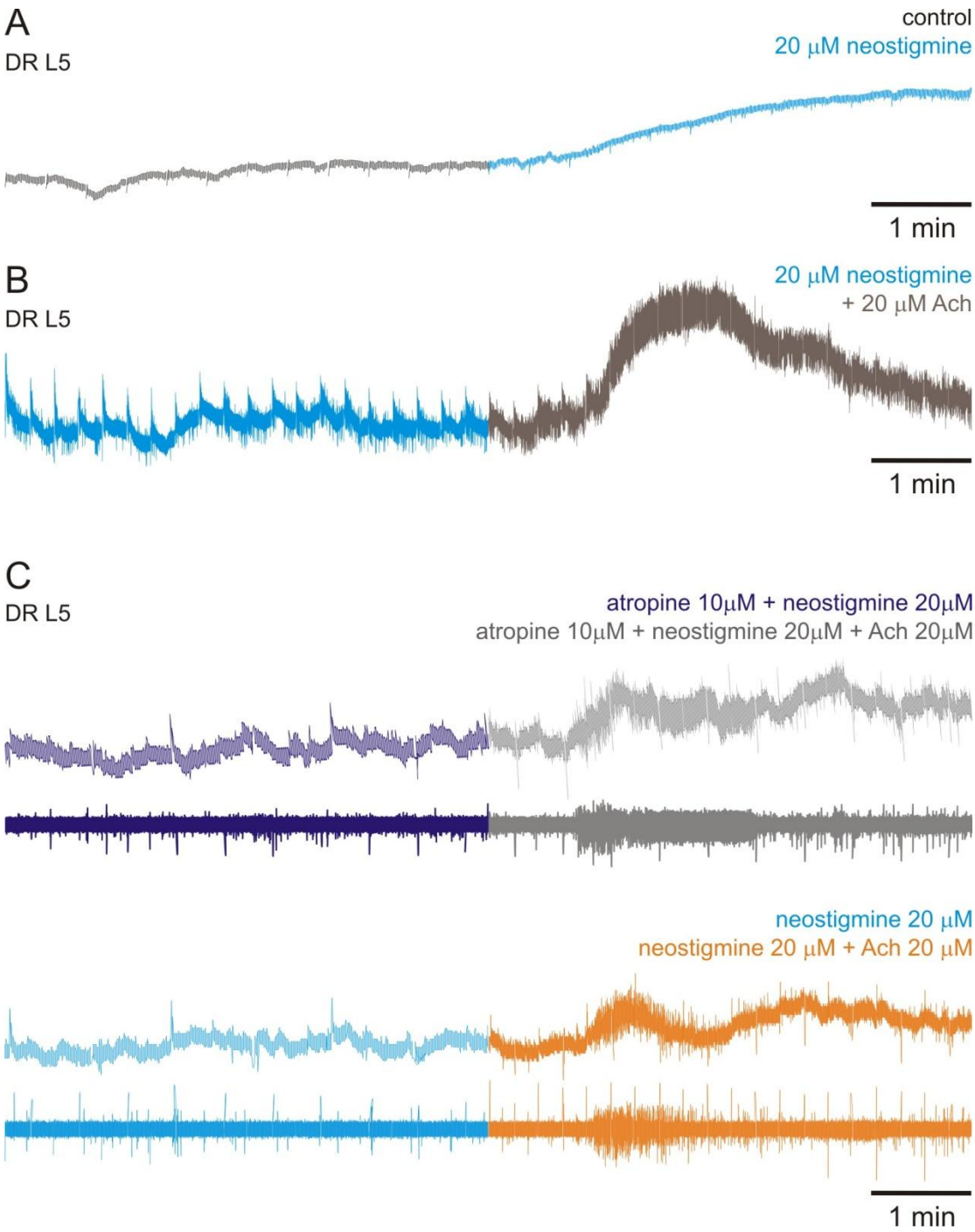
*$p<0.05$. The second application of ACh does not result in a DC depolarization of primary afferents that is significantly different from the first. However, the 3rd application does show a decrease in the amplitude of the DC depolarization of afferents.

Neostigmine led to a DC depolarization of primary afferents in some cases (30 µM: rat n=3/3; 20 – 30 µM: n=7/23 mouse Figure 24A). When seen, the depolarization likely resulted from an increase in ACh at cholinergic synapses on afferent axons. The depolarization could also be due to an increase in spinal interneuronal activity leading to a depolarization of primary afferents. However, an increase in spiking in the dorsal root was also observed in many experiments (n=3/3 rat; 30 µM, n=12/27 mouse; 20-30 µM), which could be a result of direct afferent depolarization or increased activity in interneurons contacting primary afferents.

ACh, whether applied alone (n=17 combined rat and mouse at 50-100 µM [10 values are hemicords from 5 mice]) or in the presence of neostigmine (20-30 µM; n=28/28), always caused a DC depolarization of the dorsal root axons (Figure 24B). In all but one of these occasions ACh also led to increased spiking in dorsal root axons (Figure 24C, low-pass filtered traces). The increase in background activity in the dorsal root, combined with the DC depolarization of the dorsal root, suggests that the main source of the depression of the DRP seen after application of ACh or ACh/neostigmine is due to afferent depolarization (see discussion).

As ACh could be acting on both muscarinic and nicotinic receptors, I examined the ability of ACh to lead to a depolarization in the presence of atropine. Muscarinic receptors have been identified in rodent DRG cells (Bernardini, Levey et al. 1999; Haberberger, Henrich et al. 1999), and atropine, the non-selective muscarinic receptor antagonist has been shown to block cutaneous C-fiber evoked presynaptic inhibition of the monosynaptic reflex (Yoshioka, Sakuma et al. 1990). Application of atropine (10 µM) does not affect the depolarization of primary afferents from ACh (20 µM) and neostigmine (20 µM) (56±19% of control; n=4, Figure 24C, upper panel). This is not significantly different than what is seen after ACh and neostigmine alone (see Table 8). Atropine also failed to overtly affect the increase background spiking activity seen with ACh and neostigmine (Figure 24C, filtered traces).

Figure 24 DC polarization of primary afferents by ACh/neostigmine is not due to muscarinic AChR activation



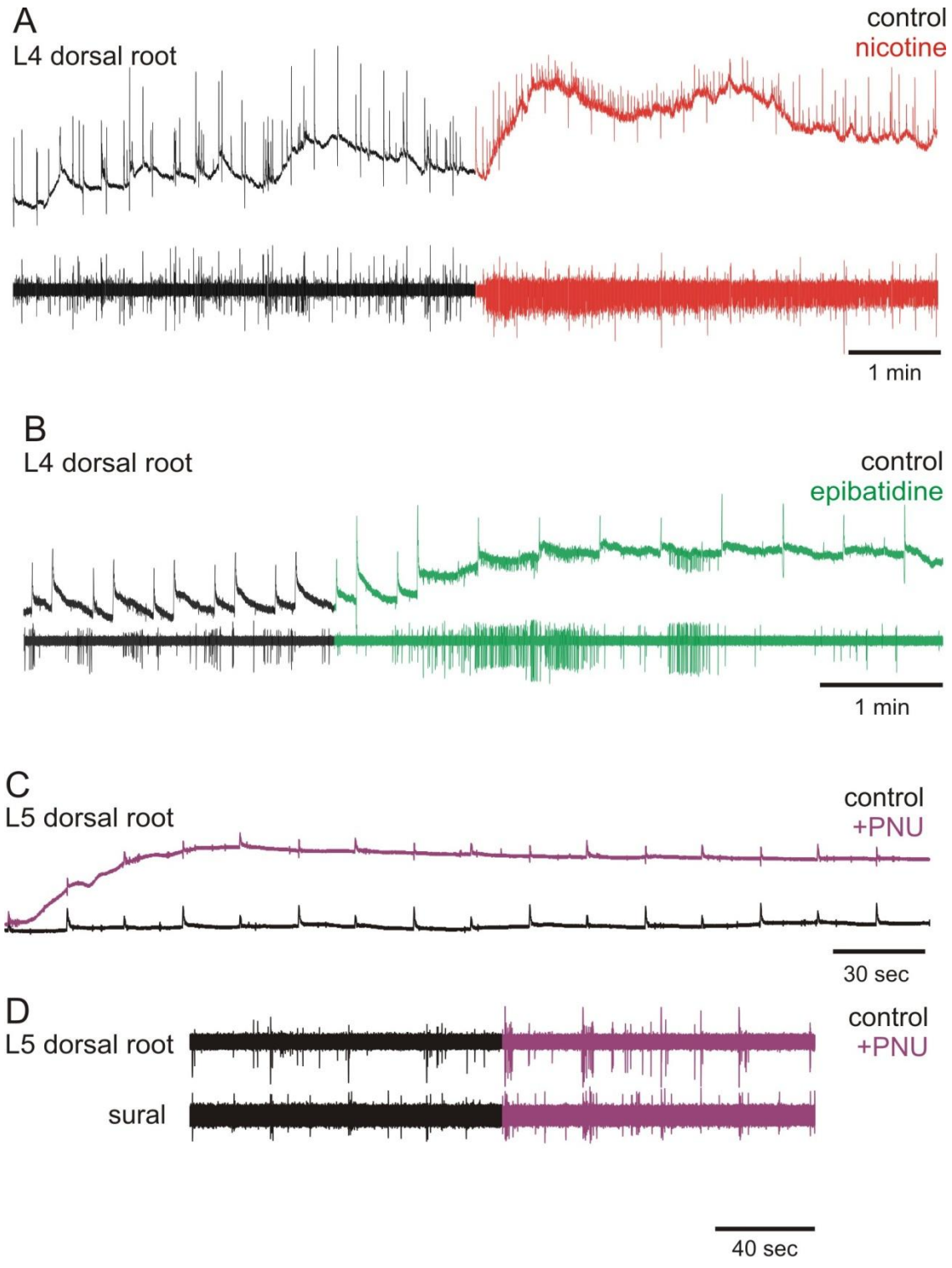
The DC polarization state of afferents was monitored in the dorsal root concurrently with stimulus evoked DRPs. In these experiments the dorsal root was stimulated at 100 μA, 100 μs every 15 seconds. The traces shown here are continuous recordings with a DC coupled amplifier to examine the effect of ACh on the membrane potential of a sample of afferents recorded by a suction electrode near the dorsal root entry zone. Drug application is marked by a color change in the trace. **A.** Neostigmine (20-30 μM) causes DC depolarization in a subset of experiments (10 of 26). **B.** Application of ACh (20-30 μM) following neostigmine led to a DC depolarization of afferents and an increase dorsal root spiking. Results are comparable in the presence of TTX (100 nM). **C.** Neostigmine + ACh depolarization is unaffected by pretreatment with atropine (10 μM). The increase in afferent spiking is likewise unaffected. Bottom traces have been low-pass filtered at 400 Hz to better display the spiking activity.

To determine if the direct depolarization of primary afferents seen after application of ACh was due to activation of nAChRs, we applied nicotine to assess its action on the polarization state of the terminals. Nicotine produced a clear DC depolarization in 2/5 cases (0.5 – 40 μM; 3 rats, 2 mouse) but unstable recording conditions made an additional 2 cases uncertain. However, an increase in DR spiking activity can also be used as a measure of the polarization state of primary afferents, and nicotine caused an increase in DR spiking (n=4/5; 500 nM – 40 μM; Figure 24A). The observed increase in spontaneous spiking activity did not appear to be as strong as seen with ACh, possibly due to its antagonist actions at α9 homomers and α9/α10 heteromers. Another explanation is the increase in desensitization rate seen with nicotine at nAChRs (Chavez-Noriega, Gillespie et al. 2000).

Epibatidine also produced a DC depolarization of primary afferents (n=4/5; 100 nM; Figure 29). Like nicotine, epibatidine also produced an increase in background dorsal root spiking (n=5/5; 100 nM, Figure 24B).

PNU-120596 caused a DC depolarization in the dorsal root (n=2/3; 1 μM; Figure 24C) and an increase in dorsal root background activity (n=3/3). This increased activity was also seen in the peripheral cutaneous nerves (n=2/3; Figure 24D). As PNU facilitates α7 activity, this observation demonstrates that α7 nAChRs can be recruited to depolarize primary afferent terminals with sufficient magnitude to produce back-propagating spikes in peripheral cutaneous afferents.

Figure 25 Nicotinic agonists the positive allosteric modulator PNU led to depolarization and increased excitability of primary afferents



Changes in the polarization state of primary afferents were monitored by a DC coupled amplifier at the dorsal root entry zone in the lumbar cord. Peripheral nerves or roots were stimulated every 15 seconds to simultaneously monitor afferent evoked responses. **A.** Nicotine (40μM) produced a desensitizing DC depolarization of primary afferents, returning toward baseline in continued presence of the drug (top panel). Results are comparable in the presence of TTX. The return to baseline in the presence of the drug is likely due to nAChR desensitization. Background spiking in the dorsal root increases on drug addition. This is shown in the AC-filtered lower panel. **B.** Epibatidine leads to a DC depolarization of primary afferent in the dorsal root (upper trace). There is also an increase in spontaneous background spiking, best seen following high pass filtering at 400 Hz (bottom panel). DC polarization changes were monitored concurrently with evoked effects. The deflections seen in the continuous traces in B are evoked DRPs, which were monitored by dorsal root stimulation every 15 seconds concomitantly monitoring DC changes in the dorsal root**. C&D.** PNU led to DC depolarization and an increase in afferent background activity. This activity increase was seen in the dorsal root and sural peripheral nerve. Traces in D have been high pass filtered at 400 Hz to more clearly see the PNU-induced increase in spontaneous background spiking.

4.4.2.1 Cholinergic depolarization of primary afferents is mediated by activation of α3, α6, and α9 containing nAChRs

d-tubocurarine depressed the DC depolarization caused by ACh to 11±4% of the depolarization seen with ACh alone (Figure 25A, top panel; n=2; $p<0.01$).The effect of d-tubocurarine on direct cholinergic depolarization of afferents was lessened when neostigmine and ACh were co-applied, with d-tubocurarine only depressing the amplitude of depolarization to 59±11% of control (n=2; data not shown), which was not considered significant due to a small sample size. Experiments using both neostigmine and ACh to cause DC depolarization did not recover on washout of d-tubocurarine (n=2). One explanation for this discrepancy could be that neostigmine permits a prolonged exposure of nicotinic receptors to ACh, and this leads to receptor desensitization/internalization or excessive Ca^{2+} loading-induced axonal injury.

Mecamylamine also depressed DC depolarization caused by ACh alone to 28± 15% of control (n=3; $p<0.05$; data not shown). The extent of reversibility of the ACh-induced DC depolarization upon mecamylamine washout is impossible to ascertain given the demonstrated depression of responses to repeated application of agonist (Table 8). Nonetheless the return in amplitude from 28±15% to 53±27% of control values suggests actions are largely reversible.

4.4.3 Nicotinic depolarization of primary afferents is independent of spinal synaptic actions

Next, we determined whether observed nAChR agonist-induced afferent DC polarizations and spiking were due to direct actions on nAChRs on primary afferents as opposed to spinal circuits. In order to show this, I examined actions in the absence of action potential dependent synaptic transmission pre-treatment of the preparation with the voltage-gated Na^+ channel blocker tetrodotoxin (TTX) and by isolating roots/nerves from spinal cord.

Application of ACh (20 μM) following neostigmine (20 – 30 μM) and TTX produced a depolarization of the dorsal root (n=13/14) (Figure 26B, top trace), confirming that a major portion of ACh-mediated depolarization is due to activation of acetylcholine receptors located directly on primary afferents. ACh and neostigmine induced DC depolarization in the presence of TTX is not different than the depolarization seen in the absence of TTX (n=5; 80±30% of control; p=0.6; data not shown). In five experiments, a dorsal root was cut away from the spinal cord to monitor background firing and polarization state when ACh was added to the bath. Remarkably, when the afferents were cut away from the spinal cord and their cell bodies, ACh depolarized the isolated roots and also increased background spiking roots (n=5/5; 50 μM mouse; Figure 26C, bottom trace). These experiments demonstrate the presence of extrasynaptic nAChRs on primary afferent axons that, when activated, directly depolarize the afferents.

When added concurrently with the voltage-gated sodium channel blocker TTX (100 nM), neostigmine is also capable of producing a DC depolarization (n=3/8; 20-30μM; data not shown). As neostigmine does not activate AChRs but reduced ACh degradation, endogenous ACh release is required for the observed DC depolarization. Variability in level of tonic ACh release from afferents may explain the inconsistency of afferent DC depolarization observed with neostigmine.

To determine whether the direct depolarization of afferents was due to activation of nicotinic and not muscarinic receptors on primary afferents, the effects of nicotine on the polarization state of the dorsal root was examined. When nicotine was applied in the presence of TTX, there was a depolarization of the dorsal root (n=2/2; 40 μM – 1 rat, 1 mouse) (data not shown), demonstrating the afferents are being depolarized through activation of nAChRs located directly on primary afferents.

4.4.3.1 d-tubocurarine and mecamylamine, but not DHβE depress the direct cholinergic depolarization of primary afferents

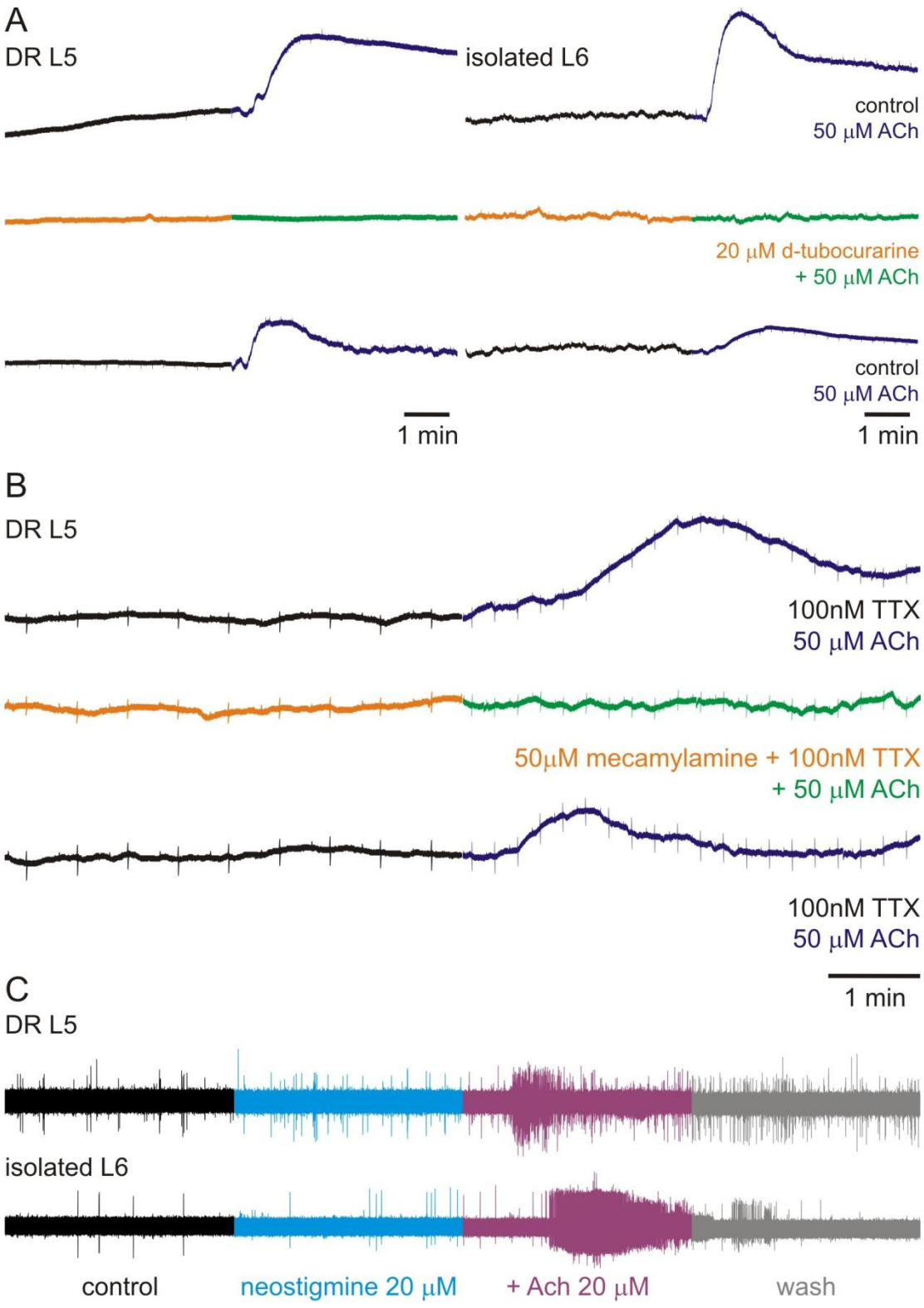
When a dorsal root was isolated from the cord, ACh also produced substantial DC depolarization that was almost completely blocked by d-tubocurarine (n=2; 7±2% of ACh-induced depolarization; $p<0.01$) and there was a partial recovery on wash to 38±6% of ACh-induced depolarization (n=2; Figure 26A, middle traces).

Mecamylamine did not cause any change in the polarization state of primary afferents on its own. However in the presence of TTX (100 nM), pre-incubation of the isolated hemicord in mecamylamine (50 μM) also appears to greatly depress the ACh-induced DC depolarization to 30 ±16% of ACh-induced depolarization (n=4 [2 mice with 2 hemicords in the same bath]; $p<0.01$; Figure 26B).

DHβE (50μM) did not block the ACh/neostigmine-induced depolarization in the presence of TTX, suggesting that α2, α3, and α4-containing nAChRs are not involved in the neostigmine and ACh-induced DC depolarization (107± 25% of ACh-induced depolarization; n=5; data not shown).

These data show that d-tubocurarine and mecamylamine sensitive nAChRs are located extrasynaptically along the afferent axons, and that activation of these receptors leads to a direct depolarization of primary afferents.

Figure 26 ACh induced direct DC depolarization is blocked by d-tubocurarine and mecamylamine



The effect of nAChR antagonists on the ACh DC depolarization was examined. Changes in the polarization state of primary afferents were monitored by a DC coupled amplifier at the dorsal root entry zone in the lumbar cord. Peripheral nerves or roots were stimulated every 15 seconds to simultaneously monitor afferent evoked responses. Drug application is denoted by a color change in the trace. **A.** ACh caused a DC depolarization in both a dorsal root in continuity with the spinal cord and a dorsal root that has been cut away from the cord. On a subsequent application of ACh after pre-application of d-tubocurarine, the DC depolarization was blocked. Following washout, a 3rd application of ACh was again able to produce a depolarization. **B.** ACh also caused a DC depolarization in primary afferents in the presence of TTX. Mecamylamine blocks this depolarization. Results were similar in the absence of TTX. Block of the ACh induced DC depolarization in the presence and absence of TTX by mecamylamine and d-tubocurarine demonstrates that the DC depolarization is due to activation of nAChRs on primary afferent axons. d-tubocurarine and mecamylamine both antagonize α3, α6, and α9 containing nAChRs, all of which are found on a subset of primary afferents. **C.** Traces low-pass filtered at 400 Hz. Recordings are from dorsal root L5 still in continuity with the spinal cord. Dorsal root L6 has been cut away from the cord and recorded to demonstrate the presence of AChRs directly on the axons. Spontaneous spiking occurs in dorsal roots that are connected to the spinal and isolated roots that have been cut away from it. There is greater spiking in the dorsal roots in continuity with the cord. Neostigmine and subsequent application of ACh lead to increased spiking in both roots.

In sum, ACh and ACh + neostigmine produce a DC depolarization of primary afferents both in the presence and absence of TTX due to direct activation of nAChRs on afferent axons. These depolarizations are largely blocked by d-tubocurarine and mecamylamine, demonstrating that the DC depolarization mediated by ACh + neostigmine is due to activation of α3, α6, and/or α9 containing nAChRs located directly on afferent axons.

4.4.4 ACh induced DC depolarization is NOT due to overlapping pharmacology with $GABA_A$ receptors

In order to assess drug actions on muscimol-induced DC depolarizations in comparison to a previous muscimol application, multiple applications of muscimol were examined to determine if the resulting depolarization changed over time (Table 9). In three consecutive applications of muscimol, there was no change in the amplitude of the depolarization when compared to the initial exposure.

Table 9 DC depolarization differences by repeated applications of muscimol

10 µM muscimol application		**Depolarization amplitude (% of control muscimol depolarization)**
2nd application	n=5	70 +/- 27
3rd application	n=5	76 +/- 31

Muscimol reversibly depolarized primary afferents when applied alone (n=15/15). The depolarization seen with muscimol remained in the presence of TTX (76±24% of muscimol-induced depolarization; n=5). As this is not significantly different from the reduction seen following control experiments on repeated muscimol application, it is likely TTX had little or no effect and that the muscimol-induced DC depolarization, demonstrating that muscimol acts largely through direct activation of $GABA_A$ receptors on primary afferents (Figure 27A).

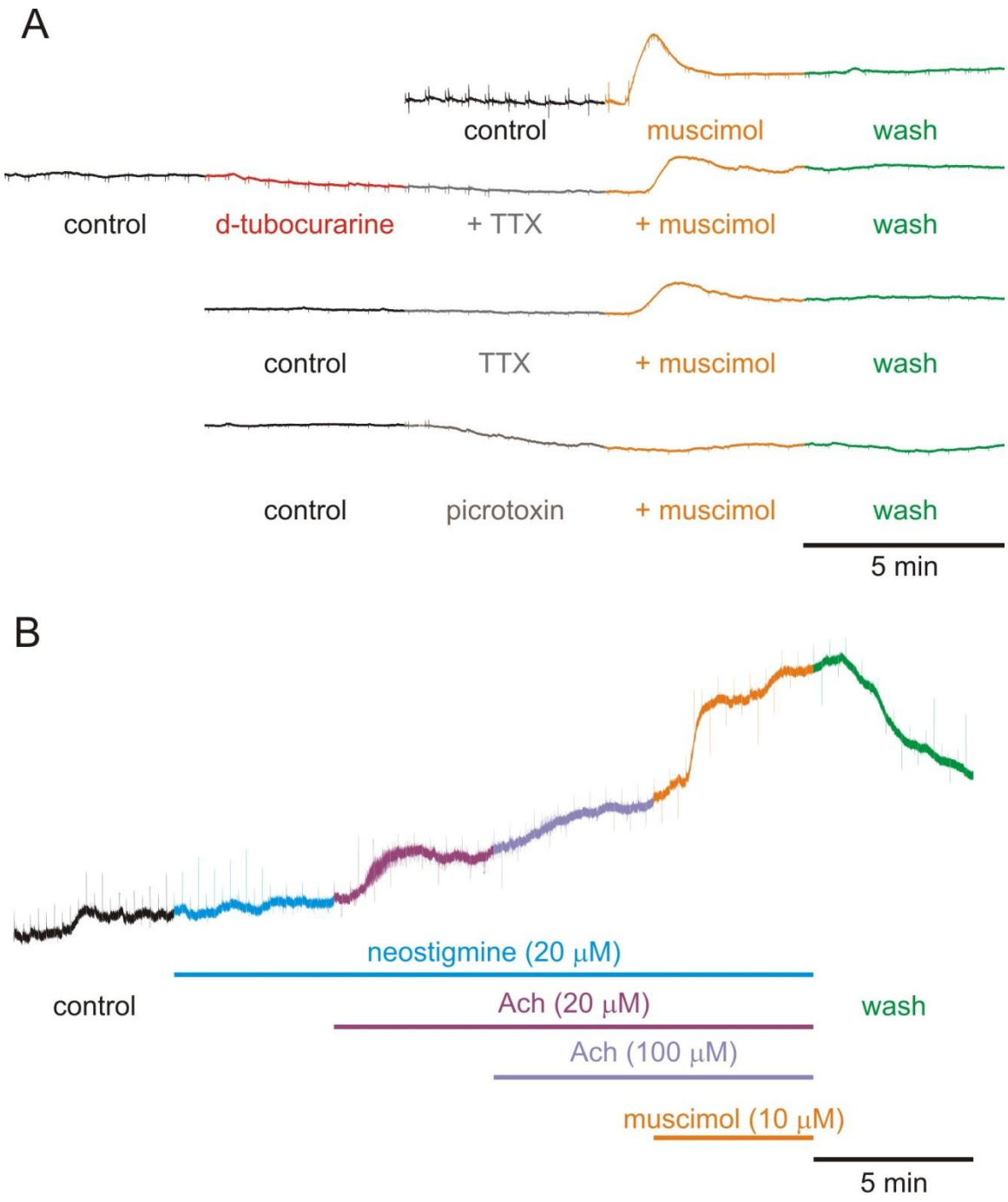
Surprisingly, application of muscimol following an ACh + neostigmine-induced depolarization still produced an additional depolarization of primary afferents (n=4/4; Figure 27B). As cation-conducting nAChRs have a more positive reversal potential than that for the chloride-conducting GABA receptor, one possibility for the continued ability of muscimol to depolarize the dorsal roots is through activation of $GABA_A$ receptors on a different subset of afferents than those affected by ACh. To my knowledge, no published studies have reported non-specific effects of muscimol, however, muscimol (10μM) does elicit currents on homomeric α7 nAChRs expressed in HEK cells (A. Jenkins, personal communication), so the possibility exists that ACh + neostigmine did not fully activate nAChRs allowing for additional nAChR activation by muscimol.

4.4.4.1 $GABA_A$R mediated PAD is not affected by d-tubocurarine

At higher doses, the nAChR antagonist d-tubocurarine has been shown to also antagonize $GABA_A$R activation (Johnston 1996). Conversely, both picrotoxin and bicuculline, traditionally used as $GABA_A$R antagonists, can act as antagonists at some nAChRs (Erkkila, Weiss et al. 2004; Gomez-Casati, Fuchs et al. 2005). I examined the possibility of overlapping pharmacological actions in both $GABA_A$ and nAChR mediated depolarization of primary afferents.

Pre-application of d-tubocurarine did not significantly affect the DC depolarization produced by muscimol in the presence of TTX (74±36% of muscimol-induced depolarization; n=5/5). This is consistent with a previous research study showing the IC_{50} of d-tubocurarine at $GABA_A$Rs to be a much higher concentration (85 μM) than the dosage I employed in these experiments (20 μM) (DeFeudis 1978). In one of these experiments, we subsequently blocked the muscimol-induced DC depolarization with picrotoxin (to 6.55% of control) clearly demonstrating that the muscimol-induced DC depolarization could be blocked by $GABA_A$R antagonists, but not by the nAChR antagonist d-tubocurarine (Figure 27A).

Figure 27 ACh/neostigmine DC depolarization is not due to overlapping pharmacological actions on $GABA_ARs$



Changes in the polarization state of primary afferents were monitored by a DC coupled amplifier at the dorsal root entry zone in the lumbar cord. Dorsal roots were stimulated every 15 seconds to simultaneously monitor afferent evoked responses. **A.** Muscimol produced a DC depolarization in the absence and presence of TTX, demonstrating direct actions on primary afferents. d-tubocurarine does not alter the DC depolarization caused by muscimol. However, muscimol induced DC depolarization was blocked by picrotoxin. Alone, picrotoxin caused a slight hyperpolarization in the membrane potential of afferents. **B.** The bars below the trace denote the presence of a given drug in the bath. Following application of neostigmine and ACh, muscimol was still able to produce a depolarization. The ability of muscimol to lead to a further DC depolarization suggests GABAergic actions on separate afferent populations.

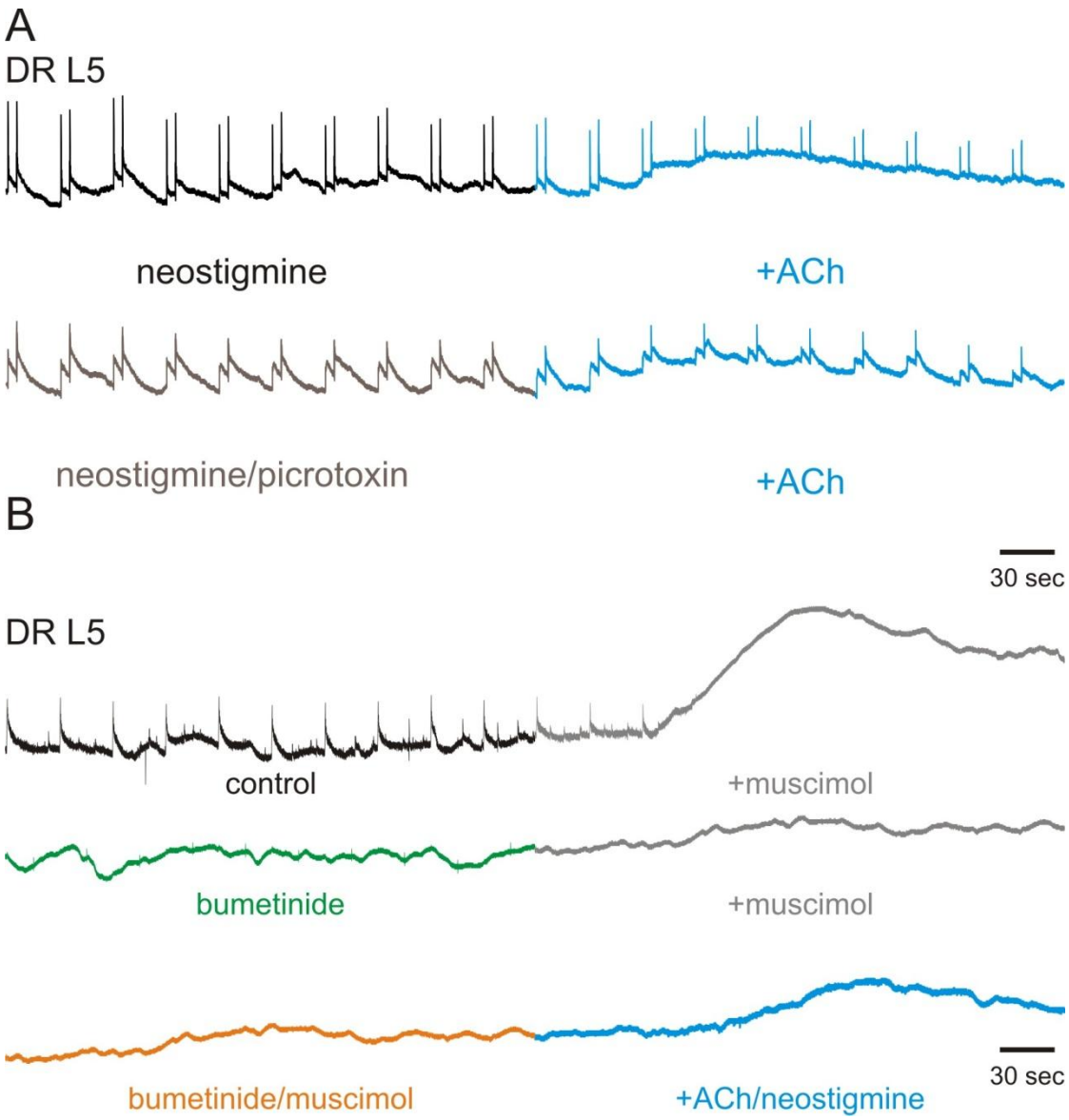
4.4.4.2 Cholinergic PAD is not affected by picrotoxin

To demonstrate that the ACh-induced DC depolarization does not have actions on $GABA_A$Rs, pretreatment with picrotoxin was done before adding ACh + neostigmine or the nAChR agonist epibatidine. In the presence of 25 μM picrotoxin, the ACh + neostigmine (n=2/2) or epibatidine (n=1/1) induced depolarization of primary afferents was unaffected (77±4% of ACh + neostigmine or epibatidine- induced depolarization; Figure 28A).

4.4.4.3 ACh + neostigmine, but not muscimol leads to a depolarization of primary afferents after collapse of the chloride gradient.

Bumetanide blocks the chloride co-transporter, NKCC1 (McGahan, Yorio et al. 1977). This transporter maintains a depolarizing chloride gradient in primary afferents (Stein and Nicoll 2003). Thus, if the chloride gradient can be collapsed to where muscimol is no longer able to produce a DC depolarization of primary afferents, a depolarization seen in the presence of ACh must be solely due to activation of ACh receptors. In two experiments (4 hemicords) application of bumetanide greatly decreased the muscimol induced depolarization of afferents (n=4; 17±9% of control muscimol depolarization; $p<0.01$). In one of these experiments (n=2 hemicords), ACh + neostigmine produced a DC depolarization in the presence of bumetanide + muscimol (n=2; 39±50% of control muscimol depolarization; Figure 28B).

Figure 28 ACh+neostigmine depolarization of afferents persists after block of $GABA_A$Rs and collapse of the chloride gradient



The polarization state of primary afferents was monitored with a DC coupled amplifier. These recordings were coincident with stimulation of the dorsal root to elicit a DRP. Stimulus induced DRPs can be seen in A. and in the control trace of B. Drug application is denoted by a change in the color of the trace. **A.** ACh (20 μM) led to a DC depolarization after neostigmine (20 μM). Addition of picrotoxin (25 μM) reduces, but does not block cholinergic DC depolarization. **B.**

Muscimol (10 μM) caused a DC depolarization of primary afferents. After bumetanide (50 μM) was applied for 3 hours resulting collapse of the chloride gradient through block of the ion co-transporter NKCC1. Application of muscimol no longer led to a DC depolarization. Subsequent application of ACh + neostigmine (both at 20 μM), however, still caused a DC depolarization further demonstrating that the ACh + neostigmine DC depolarization of afferents is independent of $GABA_A$Rs.

Though $GABA_AR$ activation produces direct depolarizations of primary afferents, these results demonstrate that neostigmine + ACh mediated DC depolarization of afferents is not due to overlapping pharmacological actions at $GABA_ARs$. The $GABA_AR$ antagonist picrotoxin does not affect neostigmine + ACh mediated DC depolarization of afferents (Figure 28A), and the nAChR antagonist d-tubocurarine likewise does not affect the muscimol induced DC depolarization of afferents (Figure 27A). Finally, collapse of the chloride gradient results in the block of GABAergic, but not cholinergic, depolarization of primary afferents (Figure 28B), further confirming cholinergic actions independent of GABA and GABA receptors. Good

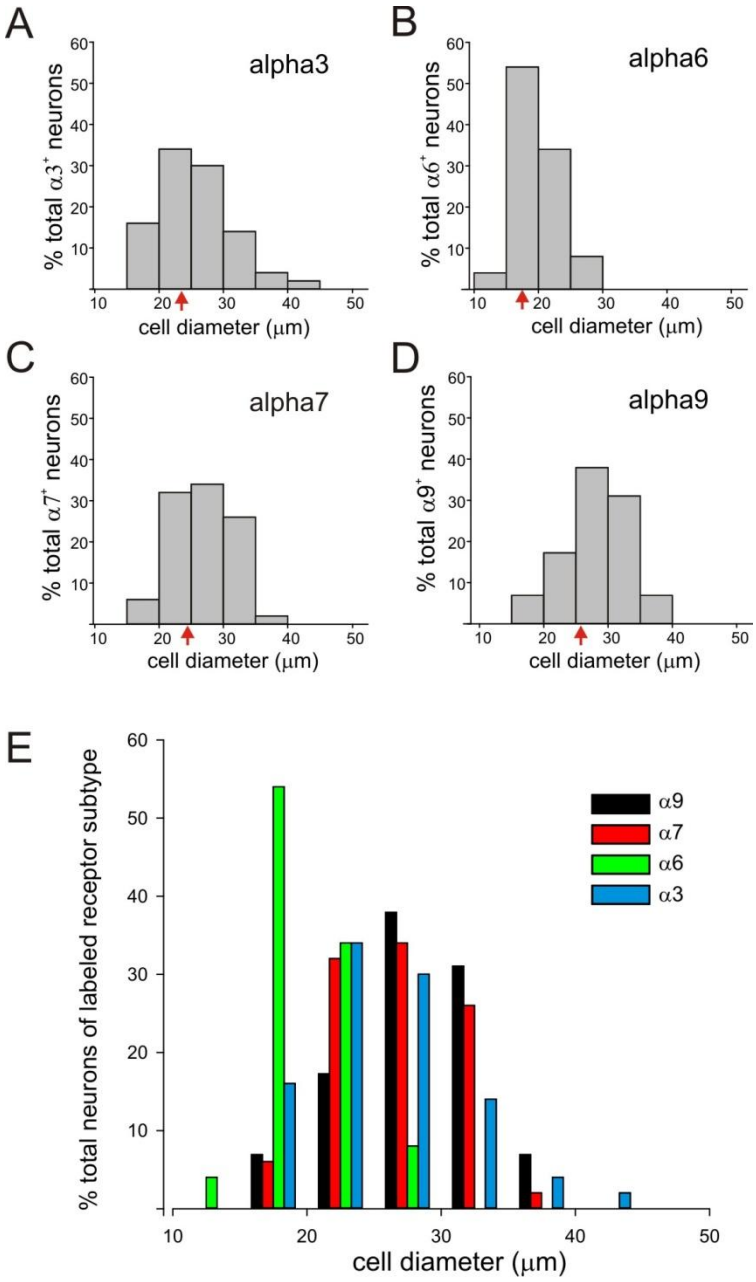
4.4.5Immunohistochemical detection of nicotinic receptors in primary afferents

Expression of α3, α6, α7 and α9 nAChR subunits were examined using fluorescence immunohistochemistry in DRG and in isolated cutaneous or muscle nerves. These receptor subunits were chosen based on a preliminary examination of expression patterns in the DRG using the Allen Brain Atlas (www.brain-map.org). Here, I saw expression of these receptors in subsets of DRG cell bodies, suggesting that they may have afferent-class specific physiological actions. Further support for a more detailed examination of these receptor subtypes came from differential effects of the nicotinic antagonists, as described above. Labeling of the nAChR subunits was done in combination with some markers commonly used to distinguish primary afferent phenotypes: calcitonin gene related peptide (CGRP), somatostatin, and parvalbumin. CGRP functions as a peptide transmitter mainly in small-diameter afferents arising from muscle, skin and viscera (Shen 2009; Shen and Colletti 2009). Somatostatin is also a peptide transmitter. It labels roughly 10% of small cell bodies in the DRG of afferents that respond to either noxious or non-noxious stimuli (reviewed in (Willis and Coggeshall 1991)). Parvalbumin is a calcium binding protein whose expression is primarily limited to large diameter, low-threshold proprioceptors in the rodent DRG (Wu, Sheng et al. 2009). Finally, I also looked at the

expression patterns of the nicotinic subunits in relation to S-100, which has been shown to label satellite cells in the DRG (Alvarez-Maubecin, Garcia-Hernandez et al. 2000), to determine if the nAChR expression was limited to neurons, or if they were also expressed in glia. S-100 has also been shown to label some DRG neurons (Hancock, Canetta et al. 2008). We distinguished between S-100^{+} glia and neurons based on cell morphology. We examined expression pattern in DRG and, when possible, in isolated nerves containing purely muscle or purely cutaneous afferents.

All 4 nicotinic receptors were detected in mouse DRG and the overall soma diameter distribution for a measured subsample is shown in Figure 29. Kruskal-Wallis One Way Analysis of Variance on Ranks detected significant differences between these populations ($p < 0.001$). Soma diameters were determined as described in the Methods.

Figure 29 Diameter of DRG neurons expressing various nicotinic receptor subunits

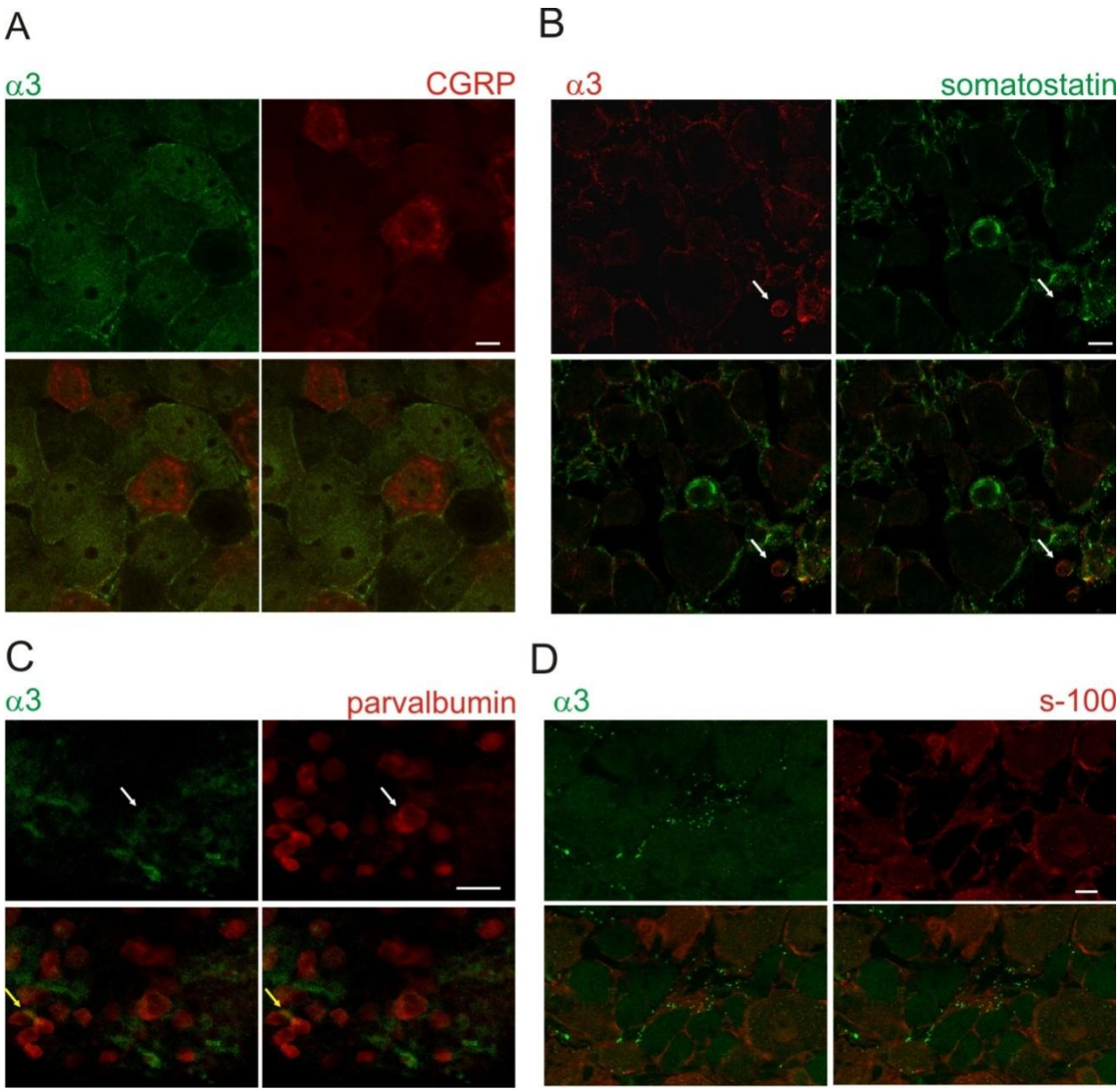


A-D. Histograms of soma diameters of α3, α6, α7 and α9 nAChR subtypes, respectively, expressed as a percentage of the total sample for each given subtypes and plotted in 5 μm bins. Mean values are identified by arrows. **E.** Combined histogram of all four populations to aid comparison.

4.4.5.1 α3-containing nAChRs

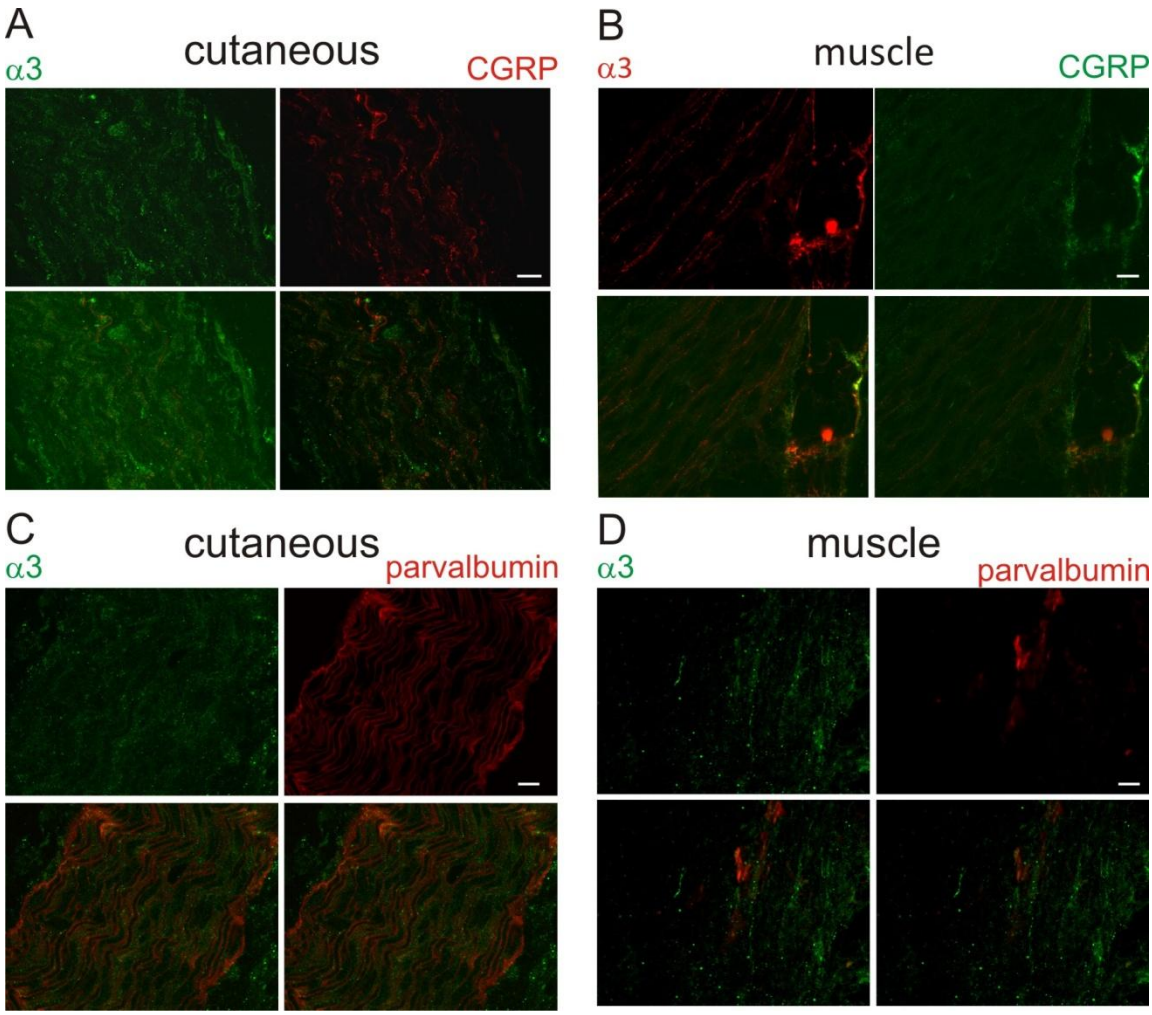
In the DRG, α3-containing nAChRs were observed to be expressed predominantly in a subset of medium-diameter afferents with soma diameters ranging from 15 to 42 µm with a mean of 23.3± 5.7 µm. The soma diameter distribution is shown in Figure 29. In the DRG, expression was dominant in the cell membrane. Expression clearly co-localized with $CGRP^+$ neurons with limited evidence of co-labeling with somatostatin or parvalbumin (Figure 30A-C). α3 and somatostatin appeared to have overlap in very small diameter cells (Figure 30B). While weak, co-expression with parvalbumin was also evident in some cells (Figure 30C). α3 expression appears to be restricted to neurons, as there is no co-labeling with S100 (Figure 30D). In isolated peripheral nerves, there is limited evidence of expression of α3 in cutaneous nerves but there is clear α3 expression in muscle nerves (Figure 31). However, in neither muscle nor cutaneous nerve was there clear overlap with CGRP or parvalbumin (Figure 31).

Figure 30 Nicotinic α3 receptor labeling in dorsal root ganglia



A subset of DRG cells with varying sizes are CGRP+. **A.** Comparison with CGRP. A small degree of overlap can be seen in the smallest diameter cell bodies. **B.** Comparison with somatostatin. There may be some overlap in smaller structures (arrows). **C.** Comparison to parvalbumin. Overlap with a small diameter neuron is shown at arrow. **D.** Comparison to S-100 labeling. No overlap is seen. In all cases, the first panel is α3 labeling followed by labeling for CGRP, somatostatin, parvalbumin or S-100. The lower two panels for A-D are merged images with different merge settings to aid in comparison of labeling patterns. A-D scale bars are 10 µm.

Figure 31Nicotinic α3 labeling in muscle and cutaneous nerves

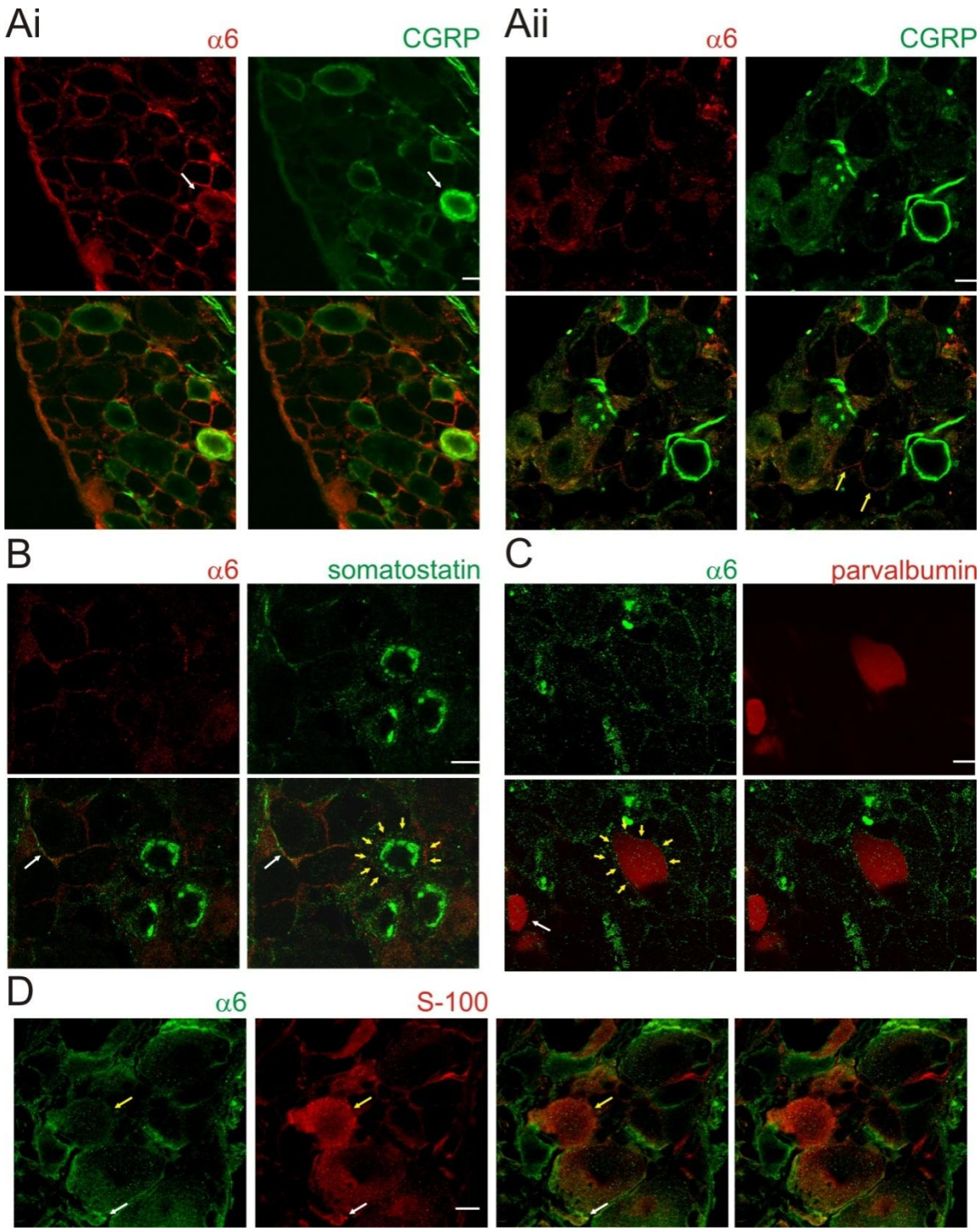


Comparison with CGRP labeling in cutaneous (**A**) and muscle nerves (**B**). There does not appear to be any overlap in expression patterns in cutaneous afferents. Comparison to parvalbumin in cutaneous (**C**) and muscle nerves (**D**). α3 puncta surround a parvalbumin positive afferent in the muscle nerve. The lower two panels for A-C are merged images with different merge settings for comparison. In D, these are the two most rightward panels. A-D scale bars are 10 µm.

4.4.5.2 α6-containing nAChRs

α6 labels a subpopulation of small DRG cells of varying sizes with soma diameters ranging from 12 to 24 µm with a mean of 17.2± 2.8 µm (Figure 32). α6+ neurons are significantly smaller than α3, α7 and α9 expressing neurons, whose cells diameters in turn are not significantly different than each other (Dunn's Pairwise Multiple Comparison Procedures). The diameter distribution is shown in Figure 29. When co-stained with parvalbumin and CGRP, there was overlapping expression in larger diameter cell bodies (Figure 32A&C). There did not appear to be any overlap with somatostatin-containing cell bodies (Figure 32B). Interestingly, α6 was expressed in some very small cells that are S-100 positive, which are presumably glia (Figure 32D). nAChR expression on glia has been previously reported in invertebrates (Smith 1980) and recently in mouse enteric glia (Smith 1980). While there was evidence of α6 labeling in both muscle and cutaneous nerves, there was no evidence of co-labeling with CGRP+ axons (Figure 33A). Evidence of co-labeling was found with parvalbumin in both cutaneous and muscle nerves (Figure 33B).

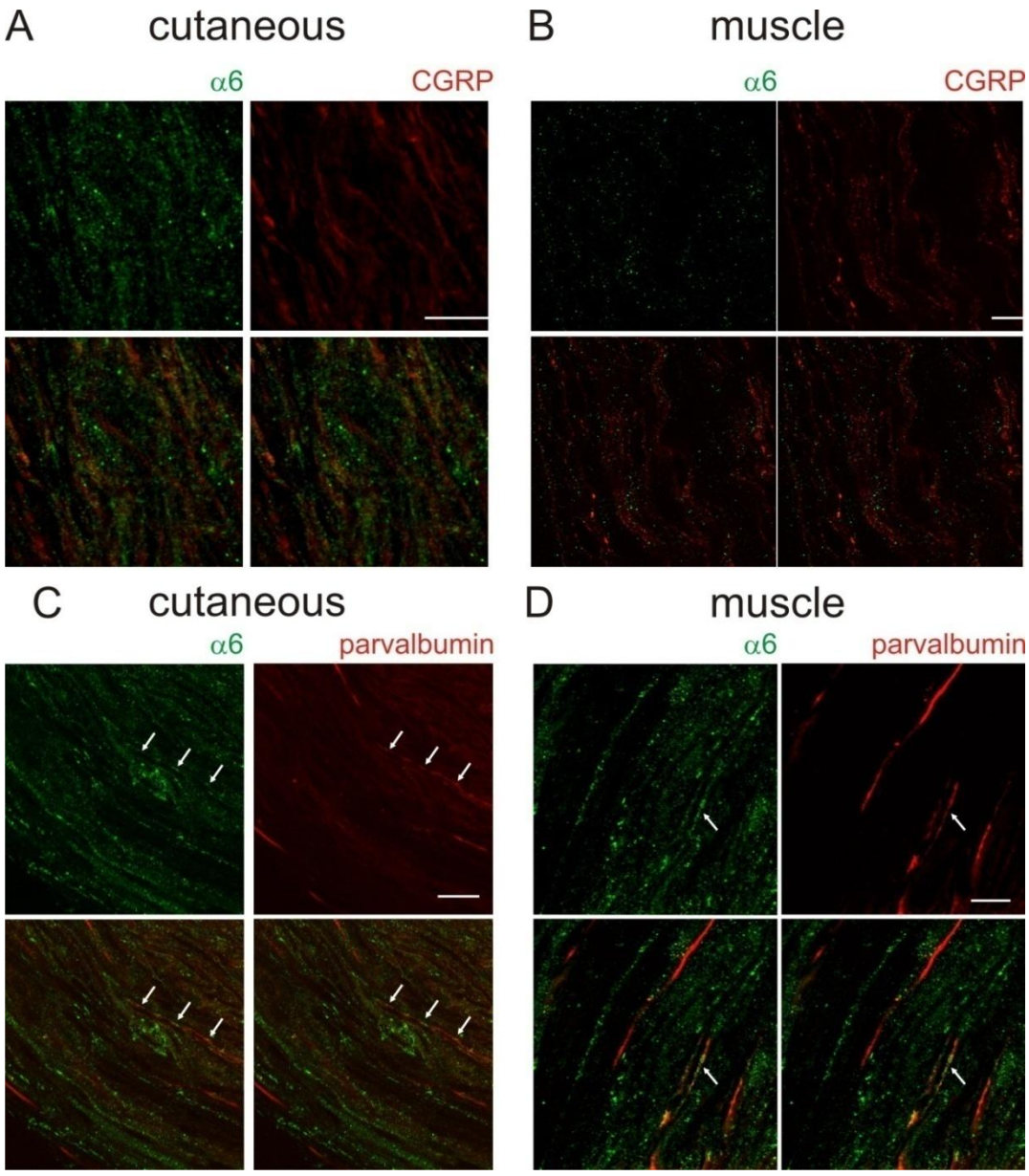
Figure 32 Nicotinic a6 receptor labeling in dorsal root ganglia



A. Comparison with CGRP. **Ai.** Note clear example of double labeled cell (white arrows top row at left). **Aii.** Different location in same animal. Note also clear a6 membrane localization (yellow arrows rightmost bottom panel). **B.** Comparison with somatostatin. Plasma membrane α6 is

apparent in a somatostatin labeled neuron (bottom right panel) as outlined with yellow arrows. Plasma membrane labeling can be found interleaved with somatostatin labeling (bottom panels white arrow) perhaps reflecting somatostatin binding to its cognate receptor in adjacent membrane region. **C.** Comparison to parvalbumin. Plasma membrane α6 labeling is associated with a parvalbumin stained neuron (yellow arrows bottom left) but not a nearby parvalbumin+ neuron (white arrow). **D.** Comparison to S-100 labeling. There is evidence of α6 labeling a small, S100 positive non-neural cell (white arrows) but not a S100+ small neuron (yellow arrows). In all cases, the first panel is α6 labeling followed by labeling for CGRP, somatostatin, parvalbumin or S-100. The lower two panels for A-C are merged images with different merge settings for comparison. In D, these are the two most rightward panels. A-D scale bars are 10 μm.

Figure 33 Nicotinic α6 receptor labeling in cutaneous and muscle nerves

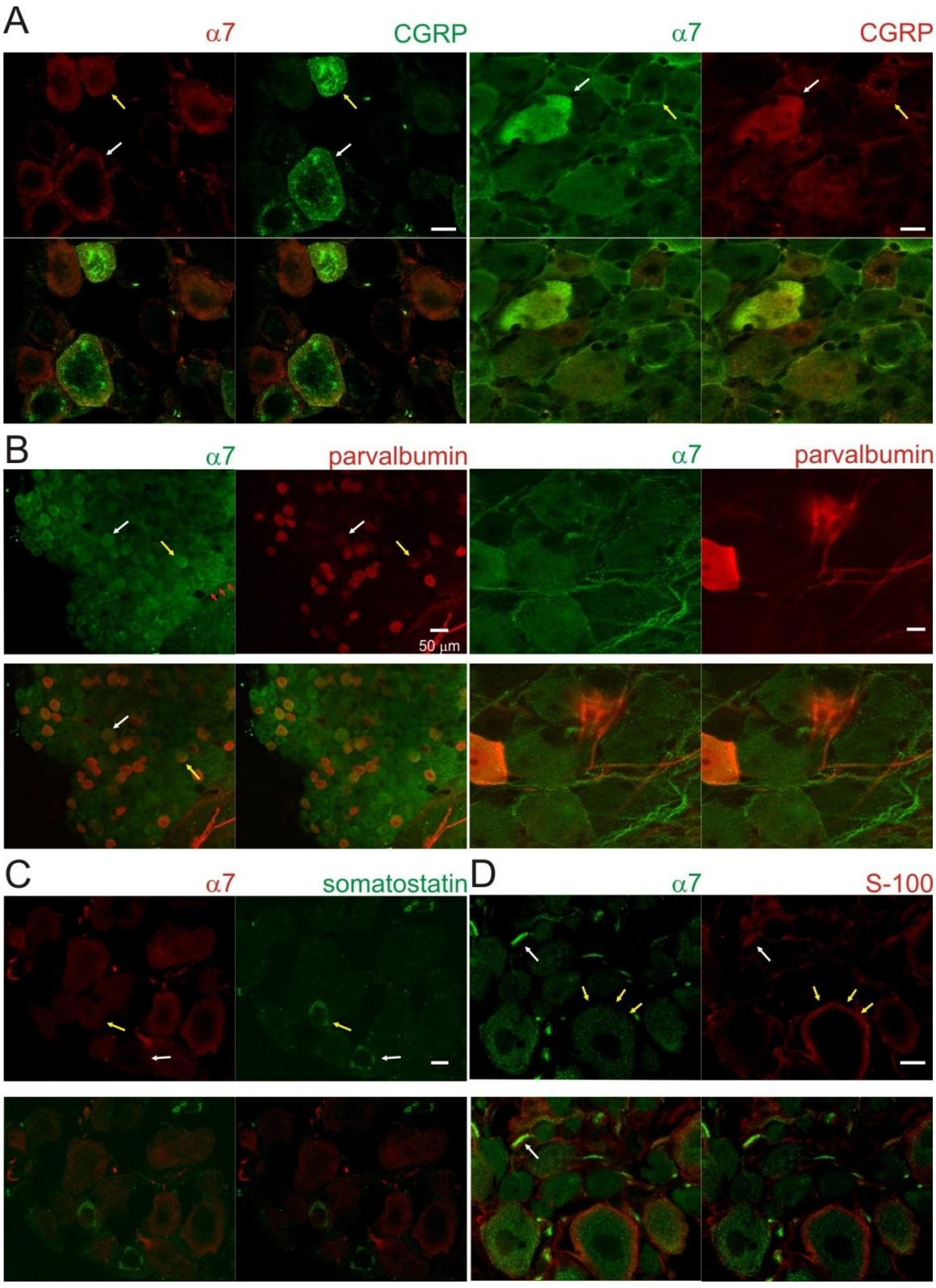


Comparison with CGRP labeling in cutaneous (**A&C**) and muscle nerves (**B&D**). While there is evidence of α6 labeling in both muscle and cutaneous nerves, there is no evidence of co-labeling with CGRP+ axons (**A&B**). Evidence of co-labeling was found with parvalbumin in both cutaneous and muscle nerves (**C& D**, white arrows). The lower two panels for A-C are merged images with different merge settings for comparison. In D, these are the two most rightward panels. A-D scale bars are 10 μm.

4.4.5.3 α7 nAChRs

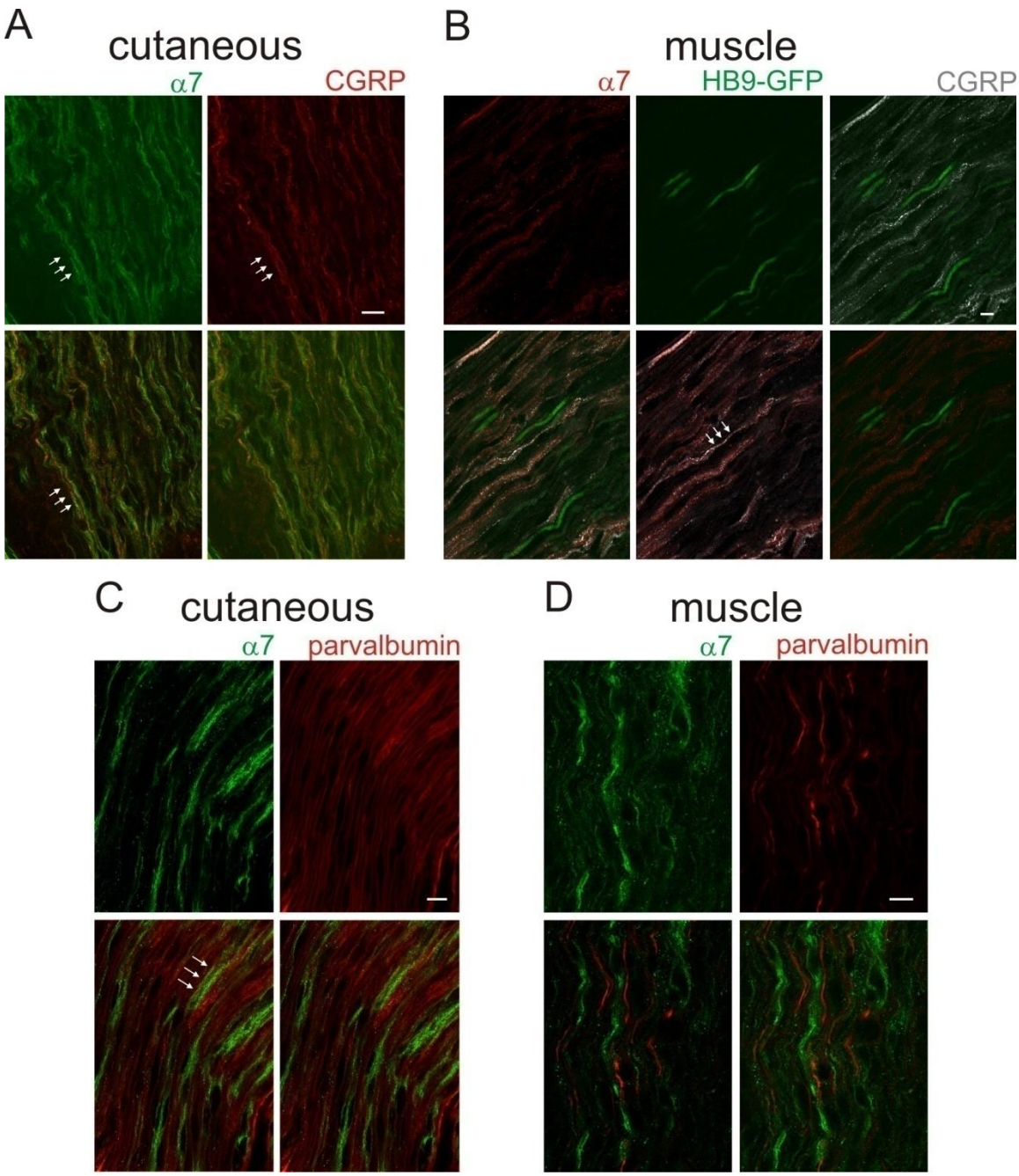
In the DRG, α7 shows labeling in a subpopulation of DRG cells, mostly of medium to large diameter cell bodies (Figure 34). Soma diameters range from 16 to 34 μm with a mean of 24.3± 4.9 μm. The soma diameter distribution is shown in Figure 29. There is some overlapping somatic expression with both CGRP and parvalbumin but no overlap in somatostatin-containing cell bodies (Figure 34A&B). Also, when co-stained for S-100 no overlap was seen in glia (Figure 34D). Most notable however, for α7 labeling is the clear intense labeling in axons (seen clearly in Figure 34B) that appears to include an S-100^{+} axon (Figure 34D). When examining α7 expression in peripheral nerves, clear co-expression is seen with CGRP in both muscle and cutaneous nerves. For the muscle nerve, motor axons were GFP+ as this was undertaken in a HB9-GFP transgenic mouse and there was no evidence of α7 or CGRP staining in motor axons. The lack of α7 or CGRP labeling in HB9 positive axons importantly verified α7-CGRP double labeling as occurring in primary afferents. For the cutaneous nerve, α7 and parvalbumin showed co-labeling in cutaneous but not muscle afferents (Figure 35).

Figure 34 Nicotinic α7 receptor labeling in dorsal root ganglia



A-C. Comparison with CGRP, parvalbumin, and somatostatin, respectively. There is overlapping expression of α7 with CGRP and parvalbumin, but not somatostatin. **A.** Co-labeling with CGRP is seen in both medium and small diameter neurons. B. For parvalbumin note that while there a subpopulation of co-labeled neuron cell bodies (right panels), there is no evidence of overlap of labeling along axons (right panels). Also note the collection of α7+ axons emanating from the DRG in panel at top left (red arrows). D. Comparison to S-100 labeling. α7 appears to co-localize with an axonal process (white arrow) and a medium diameter neuron (yellow arrows). In all cases, the first panel is α7 labeling followed by labeling for CGRP, somatostatin, parvalbumin or S-100. The lower two panels for A-D are merged images with different merge settings for comparison. A-D scale bars are 10 μm except for right set of α7-parvalbumin which is 50 μm as labeled.

Figure 35 Nicotinic α7 receptor labeling in cutaneous and muscle nerves



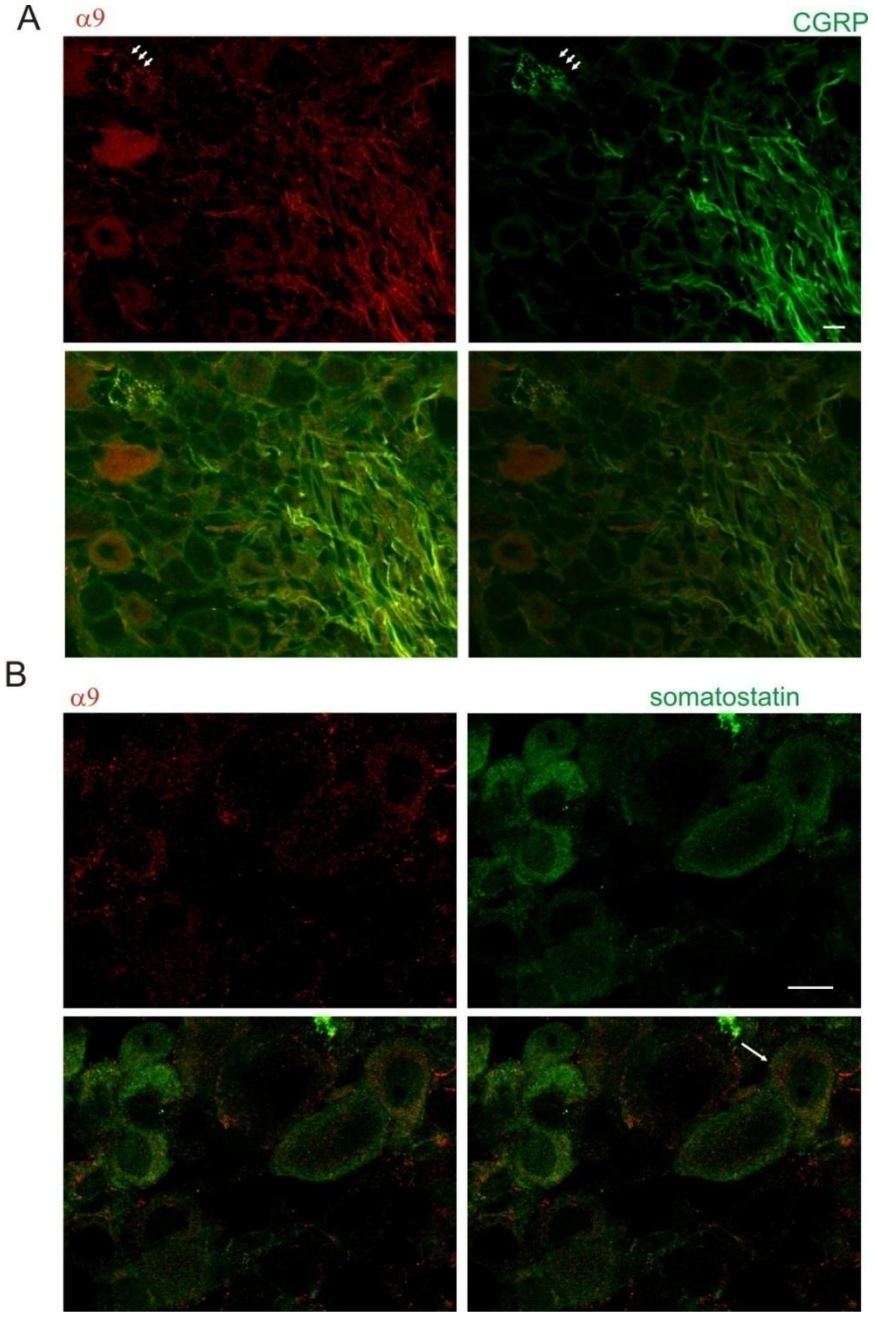
Comparison with CGRP labeling in cutaneous (**A**) and muscle nerves (**B**). Comparison to parvalbumin in cutaneous (**C)** and muscle nerves (**D**). **A&B.** Co-expression with CGRP is seen in both muscle and cutaneous nerves (white arrows). In this animal motor axons are GFP+ as this is a HB9-GFP transgenic mouse. Note that there is no evidence for expression of neither α7 nor

CGRP in motor axons thereby verifying α7-CGRP double labeling is expressed in primary afferents. **C&D.** With parvalbumin, there may be overlap in cutaneous but not muscle afferents. The lower two panels for A-D are merged images with different merge settings for comparison. A-D scale bars are 10 µm.

4.4.5.4 α9-containing nAChRs

α9 labels predominantly medium diameter cell bodies in the DRG (Figure 36). Soma diameters range from 15 to 36 µm with a mean of 25.8±5.4 µm. The diameter distribution is shown in Figure 29. There is strong overlap between CGRP and a7 receptors in axonal processes (Figure 36A). There may even be co-labeling in terminal arborizations, perhaps forming synapses on penetrating vasculature. In comparison, evidence of co-labeling with somatostatin-containing cells is uncertain (Figure 36B). α9 was not examined in peripheral nerves.

Figure 36 Nicotinic a9 receptor labeling in dorsal root ganglia



A. Comparison of α9 expression with CGRP. Note the strong axonal co-labeling including in what appears to be terminal arborizations (arrows).**B.** Comparison with somatostatin. Co-labeling with somatostatin is not obvious and weak is present (neuron at arrow). The lower two panels for A and B are merged images with different merge settings for comparison. A and B scale bars are 10 µm.

4.4.5.5 Summary of salient points regarding nAChR labeling patterns

All nicotinic subunits examined showed clear labeling in subpopulations of DRG neurons. α3, α6, and α7, were also detected in the axons of peripheral muscle and cutaneous axons. Axonal labeling of α7 and α9 showed clear overlap with CGRP, both in dorsal roots and in the periphery, demonstrating that nAChRs are trafficked to the periphery and expressed along afferent axons in support of the pharmacological effects of d-tubocurarine on ACh induced DC depolarization of isolated afferent axons. In contrast, α6 labeling tends to be more associated with the DRG cell membrane in combination with parvalbumin. While some overlap is seen with CGRP positive cells in the DRG, co-labeling was not observed in peripheral axons of muscle or cutaneous nerves. Finally, α3 nAChR subunits localized to small diameter cells of the DRG and small diameter axons in the periphery in both muscle and cutaneous nerves. α3 labeling was not regularly seen in combination with either parvalbumin or CGRP, suggesting that α3 expression is on a distinct population of afferents from the other subunits that were examined.

4.5 DISCUSSION

This study first undertook a physiological and pharmacological investigation of nAChRs in the production of afferent evoked DRPs, EFPs and DRRs, with a particular focus on cutaneous afferents. Next, I examined the involvement of nAChRs located extrasynaptically along primary afferent axons and their ability to cause a direct depolarization of those afferents. While the main focus was on nicotinic AChR contributions, I also briefly examined the contribution of $GABA_A$ receptors to these phenomena, as DRPs have long been assumed to be purely $GABA_A$ergic, and additionally because there is a great deal of overlapping pharmacology between the ionotropic cholinergic and GABAergic transmitter systems. Finally, immunohistochemistry was performed to correlate expression patterns of nicotinic receptor subunits with physiological findings.

I found that α9 nAChRs were involved in generation of both A- and C-fiber components of the DRP, as well as transmission from slower conducting A-fiber primary afferents, as measured by the EFP. Additionally, I found that α9 nAChRs were also involved in generation of DRRs. Secondly, I demonstrated that α3, α6, and α9 containing nAChR activation resulted in a direct depolarization of afferent axons. nAChRs, particularly α7 receptors, were also found to be involved in spiking activity in primary afferents and that potentiation of the α7 nAChRs also leads to a depolarization of afferents. Finally, I showed that α3, α6, α7, and α9 containing nAChRs are expressed in distinct populations of DRG neurons and that they are trafficked along afferent axons both centrally and peripherally.

4.5.1 Cholinergic effects on afferent evoked DRPs, EFPs and DRRs

DRPs were evoked by peripheral cutaneous nerve or dorsal root stimulation at strengths sufficient to recruit A fibers or both A and C-fibers. EFPs were recorded by microelectrodes in the deep dorsal horn in a region where cutaneous afferent stimulation resulted in the largest signal. When primary afferents are depolarized to above threshold, DRRs are generated and propagate both

ortho- and antidromically along the length of afferent axons. I examined the effects of nicotinic receptor antagonists on DRRs generated from afferent stimulation. Interestingly, the majority of spontaneous spiking events precede the DRP.

4.5.1.1 Atropine block of muscarinic AChRs does not depress the DRP, but it does show facilitation on washout

I first examined the metabotropic AChR contribution to the DRP. The metabotropic AChR antagonist atropine did not affect the cutaneous afferent-evoked DRP during drug application. On washout of atropine, there was an increase in the overall area of the DRP, and there was also a facilitation of the peak amplitude of the C-fiber component of the DRP. Rebound facilitatory actions on washout may have been due to plastic alterations in signal transduction pathways after extended metabotropic receptor block. Based on the response seen, these receptors may be preferentially located on circuits associated with C-fiber afferent. Previous reports demonstrate a muscarinic contribution to depression of synaptic transmission from afferents terminating in the superficial dorsal horn (Kling 2011). As there is evidence for M2 and M4 muscarinic receptors in DRG (Haberberger, Henrich et al. 1999; Tata, Tripiciano et al. 2000), these G_i-coupled receptors may serve an inhibitory role for muscarinic transmission onto primary afferents. This raises the possibility that activation of muscarinic receptors contribute to a balance between excitatory and inhibitory tone in primary afferents. Block of muscarinic AChRs could have led to a compensatory change in the excitability of the circuitry responsible for generating DRPs. It is possible that atropine's actions were due to non-specific interactions at nAChRs (Roskam and Koch 2009; Uysal, Mizuno et al. 2009) or $GABA_A$Rs (Ettema, Zhao et al. 2006; Danielyan, Zellmer et al. 2009), and indeed, after application of mecamylamine, there was a significant facilitation of the DRP on drug washout (Table 4). However, non-specific interactions with nAChRs would have likely also led to a reduction of the DRP, which was not observed with atropine.

4.5.1.2 Nicotinic Antaonist effects on afferent evoked DRPs, EFPs and DRRs

I found that α9 nAChRs contribute to generation of the DRP. I also provide evidence supporting the role of α6 containing nAChRs specifically in generation of the C-fiber component of the DRP. Several nicotinic antagonists were able to depress the DRP. d-tubocurarine, α–bungarotoxin, and to a lesser extent, mecamylamine, depressed the DRP. d-tubocurarine is a broad spectrum nAChR antagonist, acting at α1, α2, α3, α4, α6, α7, α9, and α10 containing nAChRs. α-bungarotoxin is a largely irreversible antagonist at α7, α9, and α10 containing nAChRs, and mecamylamine antagonizes α3, α6, and α9 containing nAChRs by open channel block (Verbitsky, Rothlin et al. 2000; Alexander, Mathie et al. 2007). These antagonists jointly act at α9 and/or α9/α10 nAChRs, though there is also the possibility of some actions at α3 and α6 containing nAChRs, where mecamylamine and d-tubocurarine have overlapping actions, but not α-bungarotoxin. Given the large depressant actions on the DRP of d-tubocurarine (peak amplitude to ~60% of control) and α–bungarotoxin (peak amplitude to ~70% of control) compared to mecamylamine (area to ~90% of control), it would appear that α9 receptors are not the only nicotinic receptors implicated in production of the afferent evoked DRPs. However, it may be that the lessened effect of mecamylamine on the DRP is because its IC_{50} value at α9 homomers is ~10 μM and my application of 50 μM may have too low to result in complete channel block at the receptor. For example, if the α9 nAChRs that mecamylamine is acting on are located mainly in the deep dorsal horn, the concentration at the synapse will likely be much lower than that applied to the bath, due to a diffusion gradient. If one assumes a gradient of 1/10 (Murray, Stephens et al. 2011) concentrations of mecamylamine may have been insufficient to provide complete block of α9 receptors in the deep dorsal horn, even in the hemisected preparation, especially given the short application time (5 minutes) in over half of the experiments where mecamylamine was applied. It is also not known if mecamylamine acts at α9/α10 heteromers, which have been found in DRG neurons in rat (Lips, Pfeil et al. 2002) and

whether mecamylamine would act with equal efficacy and potency at these heteromeric receptors. Further support *for* the involvement of α9, and α10 containing nAChRs in DRP production was demonstrated by the fact that the antagonists DHβE (α2, α3, and α4) and MLA (α7) did not have an effect on the DRP. Additionally, I noted that α-bungarotoxin showed a partial recovery of the DRP on washout of the drug, possibly due to α-bungarotoxin's reversible antagonism on some nAChRs, but not others. It was initially shown in chick that α-bungarotoxin showed reversible antagonism of the nAChR agonist, carbachol, at cochlear hair cells (Fuchs and Murrow 1992), which were later discovered to be α9/α10 nAChRs (Elgoyhen, Johnson et al. 1994). However, at other nAChRs, α-bungarotoxin is considered an irreversible antagonist.

In the presence of d-tubocurarine, the A- and C-fiber components of the DRP showed similar levels of depression. Mecamylamine, however, has preferential actions on the C-fiber component of the DRP (Table 5). Mecamylamine is a potent inhibitor of α6β4 nAChRs (IC50=0.5 μM) (Hancock, Canetta et al. 2008), and immunolabeling data showed a preferential expression of α6 receptors on small diameter DRG neurons, typically correlating to unmyelinated fibers. In addition, d-tubocurarine also acts on α6 receptors and has depressant actions on C-fiber-evoked DRPs (Figure 19A&B).

As also shown in Figure20, both d-tubocurarine and mecamylamine appeared to depress DRRs at least partly via mechanisms independent of those generating the DRP. This was also seen with α-bungarotoxin (Figure 20B). Since all are antagonists at α9 subunit-containing nAChRs, and DRG neurons containing α9 receptors have the largest mean soma diameters, it seems likely that the A-fiber component of observed DRRs are due to afferent-evoked actions of α9 receptors. The physiological implications of an A-fiber evoked A-fiber DRR that is independent of mechanisms generating the classical slow DRP will be discussed below.

As shown with the immunostaining, α3 and α7 nAChRs are found on largely medium diameter cells, which would support a role for contributions to A-fiber DRRs and/or DRPs. As stated above, neither and MLA (α7) nor DHβE (α2, α3, and α4) altered the afferent evoked DRP, suggesting these receptors are activated by pathways not recruited by stimulation of primary afferents (e.g. descending systems).

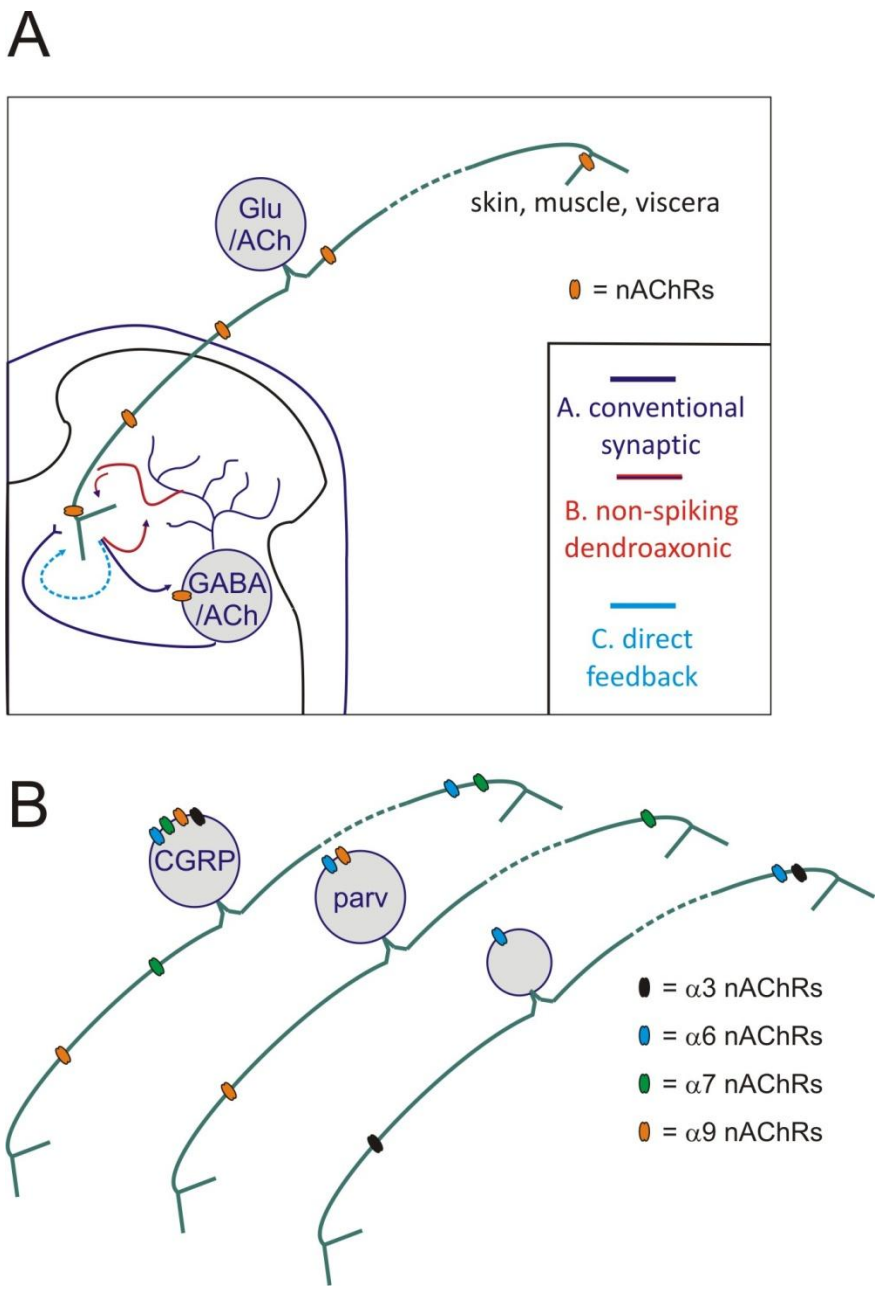
There are several possible sites of action for nicotinic antagonists in depressing the evoked DRP, These may include nAChR block on primary afferent terminals or on the spinal circuitry responsible for the generation of spontaneous DRPs and spiking events. If the action of d-tubocurarine and/or α-bungarotoxin is presynaptic, it would suggest that cholinergic interneurons in the cord are acting at primary afferent terminals to cause spontaneous spiking and DRPs. Anatomical studies have identified cholinergic interneurons that contact primary afferents (Ribeiro-da-Silva and Cuello 1990). Their cell bodies are located in lamina III, and they have dense rostro-caudal projections. Recently, a subset of these cholinergic interneurons has been shown to have repetitive discharge pattern in response to current injections (Mesnage, Gaillard et al. 2011). Repetitive firing in response to a single input would make these interneurons well suited to contribute to a long-lasting phenomenon such as the DRP. Another possibility could be the release of ACh from afferents onto other afferent terminals. A post-synaptic site of action would also require ACh release from primary afferents, and in Chapter 2, I present evidence for synthesis and packaging of ACh in primary afferents. As changes in the DRP cannot distinguish between these sites of action, both pre- and post-synaptic sites of action for d-tubocurarine, α-bungarotoxin, and mecamylamine are possible. However, the depression seen in DRRs in the presence of cholinergic antagonists that precedes any change in the DRP suggests a presynaptic mechanism of nAChRs involved in spike generation in afferents.

EFPs were monitored in the deep dorsal horn at the depth where the largest signals were elicited by cutaneous afferent stimulation. EFPs are a measure of monosynaptic transmission from

primary afferents onto spinal interneurons. The earliest portion of the EFP corresponded to A-fiber transmission and was often separable into two components, representing faster and slower conducting A-fibers. Both d-tubocurarine and α-bungarotoxin caused a depression of EFPs, d-tubocurarine depressing peak amplitude, area, and initial slope and a-bungarotoxin depressing the area. d-tubocurarine showed a preferential block of the second A-fiber field and α -bungarotoxin showed this trend (Figure 21). A depression of monosynaptic field potentials indicates a reduction in synaptic transmission to interneurons in the vicinity of the intraspinal electrode. While measuring EFPs cannot distinguish between pre- or post-synaptic mechanisms, previous studies have demonstrated a case for depression of EFPs being the result of PAD (Riddell, Huber, 1995). Depression of the EFP does, however, demonstrate that there is a depression of transmission at the first synapse from primary afferents entering the spinal cord particularly of slower conducting A-fibers, supporting the role in nAChRs located either on primary afferent terminals or on spinal interneurons receiving input from these fibers (Figure 37A).

DRRs are likely due to nicotinic receptor-mediated spikes that are mechanistically associated with the DRP or spiking in dorsal roots generated centrally distinct from, and with faster kinetics than, generation of the DRP. Depression of DRRs in the presence of nAChR antagonists could mean that DRRs are largely mediated by activation of nAChRs on primary afferent terminals which precede a DRP consisting of both nicotinic and GABAergic components. Production of DRRs and the DRP could even be mediated by the same population of spinal interneurons, as it is known that ~25% of GABAergic terminals presynaptic to primary afferents axons also contain ACh (Ribeiro-da-Silva and Cuello 1990; Alvarez 1998), such that co-release of these transmitters could elicit both fast spiking in the dorsal root, as well as a slower DRP. Interneurons with a GABAergic and cholinergic phenotype are also directly contacted by primary afferents, which would ideally position them to be intricately involved in control of afferent transmission. nAChR antagonist actions on afferent evoked actions are summarized in Table 10.

Figure 37 Models for nAChR involvement in PAD and nAChR distribution on primary afferents



A. Proposed model of cholinergic contribution to the DRP and DC depolarization of afferents. The schematic shows primary afferents projecting from the periphery into the dorsal horn of the spinal cord. Cell bodies for sensory afferents are located in the DRP. DRPs could be generated

by **A**) excitation of cholinergic interneurons (or pathways with last-order cholinergic interneurons) which in turn synapse onto primary afferent terminals, leading to depolarization of the terminal and DRP production, **B**) primary afferents synapsing onto cholinergic interneurons at a dendroaxonic synapse, where the postsynaptic dendrite is depolarized and the depolarization spreads to a nearby synapse from that dendrite back onto primary afferent terminals, or **C**) direct activation of primary afferent terminals through a release of ACh from afferents and transmitter diffusing to extrasynaptic nAChRs. Results demonstrate nAChRs along afferent axons, as well as evidence for ACh as a transmitter released from primary afferents. This provides additional sites of modulation of sensory signaling by nAChR activation; along the axons, themselves, or post-synaptically on spinal interneurons. **B.** The schematic summarizes immunohistochemical results for nAChR distribution on primary afferent axons and cell bodies. α3, α6, α7, and α9 all show expression in CGRP+ neurons, with α6 and α7 also showing in expression in the periphery on both muscle and cutaneous nerves. α9 and α6 are expressed in parvalbumin+ DRG neurons. α3 and a6 are expressed in small diameter neurons/axons both in DRG and in the periphery and muscle and cutaneous nerves. While nAChRs appear to be present both in the cell bodies of DRG neurons, as well as in axons in the periphery, it is of note that α7 and α9 show particularly strong labeling in axons, suggesting that they may be trafficked in larger amounts both centrally and peripherally.

4.5.1.3 Nicotinic agonist depress afferent evoked DRPs and EFPs through depolarization block of afferent signaling

Nicotinic agonists show similar actions on the evoked DRP, but their actions on spontaneous afferent spiking and the polarization state of primary afferents suggests a different mechanism of action. ACh, nicotine, and epibatidine, alone caused a decrease in DRP amplitude and area. Also, stimulation of cutaneous nerves in the presence of ACh and neostigmine showed a significant depression of the A- but not C-fiber component of the cutaneous nerve evoked DRP. Epibatidine didn't show a difference in A- and C-fiber components of the DRP. The decrease in the DRP seen by co-application of ACh and neostigmine is much larger than that seen by agonists alone. This discrepancy, is probably largely due to, a) block of ACh hydrolysis at nAChRs, which is the reason current decay begins quickly at cholinergic synapses (Giniatullin, Nistri et al. 2005) and b) the increased desensitization rates of nearly all nAChRs in the presence of nicotine (Chavez-Noriega, Gillespie et al. 2000) and epibatidine (Genzen, Van Cleve et al. 2001). Another possibility for the decreased effect seen with epibatidine and nicotine in comparison to ACh and neostigmine could be muscarinic involvement or also through the unconventional pharmacology of the α9 receptor. Though both nAChR agonists and antagonists have a similar effect on DRP amplitude and area, it is likely that the DRP reduction in response to agonists is due to afferent depolarization, such that when evoked, the DRPs are reduced in size, as the afferents in their depolarized state are closer to the reversal potential of the DRP. This depolarization can be seen in both DC recordings of the polarization state of primary afferent axons and evidenced by an increase in spontaneous firing in the dorsal root, suggestive of fiber depolarization.

EFPs in the presence of ACh showed a significant depression of the second component of both the A-and C-fiber field during dorsal root stimulation. ACh and neostigmine in combination equally decreased both A-fiber components of the EFP resulting from cutaneous afferent

stimulation, which corresponds well with the presence of various nAChRs in cutaneous peripheral nerves. During dorsal root stimulation, neostigmine/ACh depressed both A- and C-fiber components of the EFP. On wash, the A-fiber component showed a reversal to control values, but the C-fiber EFP did not. This discrepancy may be due to longer lasting actions on C-fibers, possibly due to an inability of nAChRs located on C-fibers to recover from desensitization, as different subunit combinations have varying rates of recovery from desensitization (Fenster, Rains et al. 1997).

4.5.2 ACh-induced DC depolarization is distinct from GABAergic PAD

ACh, ACh and neostigmine, epibatidine and nicotine were all able to produce a DC depolarization in primary afferents. Neostigmine produced PAD in a subset of experiments. Interestingly in experiments done in rat (n=3), neostigmine always caused a DC depolarization of the dorsal root. It is possible that these results demonstrate a species specific response to ACh, though the sample size is small. Rats may have an increased cholinergic tone in the spinal cord, or they may have a lower endogenous level of cholinesterase, such that the same concentration of neostigmine has a more pronounced effect. Many experiments were done in both rats and mice, and in general, results were consistent between the species. However, in the case of neostigmine, DC depolarizations were consistently produced in the rat preparations (3/3), while in mouse, the ability to chances of neostigmine producing PAD were lower (7/23). It is possible that rats have an increased cholinergic tone in the spinal cord, or they may have a lower endogenous level of cholinesterase, such that the same concentration of neostigmine has a more pronounced effect. Along with PAD, an increase in spontaneous spiking events in the dorsal root was consistently observed with all agonists, supporting a depolarization of the afferents.

With the exception of epibatidine, which was not tested, they continued to produce PAD in the presence of TTX. This demonstrates direct actions of ACh and nicotinic agonists on primary afferents. PNU-120596 also produces PAD, pointing to a contribution in nAChR-mediated PAD

through activation of a7 receptors. It is interesting the neostigmine, in some cases, was able to depolarize primary afferents in the presence of TTX. This suggests that ACh is being released in the absence of neuronal firing. Two possible sources of spontaneous ACh release are from spinal interneurons or primary afferents themselves.

All depolarizations with cholinergic agonists were accompanied by an increase in spiking in the dorsal roots, and occasionally in the periphery. Even in the absence of DRPs, increased spiking in the dorsal roots was seen, which is consistent with activation of nicotinic receptors along afferent axons. Increased spiking in the dorsal root was not seen during muscimol application. Nicotinic receptors are cation conducting channels with reversal potentials near 0 mV with relatively fast kinetics compared to $GABA_AR$ mediated receptor activity. The reversal potential implies that spikes could be produced in the absence of an additional depolarization in the axon, such as a DRP. nAChRs are able to open ~10 times quicker than $GABA_ARs$ (Maconochie, Zempel et al. 1994; Chen, Zhang et al. 1995).While the rate of desensitization in most nAChRs (Auerbach and Akk 1998) - with the exception of α7 nAChRs - is slower than in $GABA_ARs$ (Jones and Westbrook 1995), hydrolysis of ACh by AChE at the synapse occurs an order of magnitude faster than desensitization of $GABA_ARs$ (Giniatullin, Nistri et al. 2005), leading to longer lasting responses in $GABA_ARs$ with greater current transfer.

α7 nAChRs are, at least partially involved in generation of spontaneous spiking activity in the dorsal roots, and potentiation of these receptors can increase spiking activity in cutaneous afferents in the periphery.

A depression of ACh and neostigmine induced depolarization of afferents was unaffected by muscarinic receptor block with atropine, demonstrating the depolarization occurred through activation of nAChRs. ACh-mediated depolarization of afferent was, however, greatly depressed

by d-tubocurarine and mecamylamine, but not by DHβE, suggesting the involvement of α3, α6, and α9, continuing nAChRs.

A summary of antagonist and agonist actions on neostigmine/ACh mediated DC depolarization of afferents can be found at the bottom of Tables 10 and 11.

Both the DC depolarization of afferents and DRP production are generally thought to result from $GABA_AR$ activation on primary afferent terminals. However, $GABA_ARs$ and nAChRs have overlapping pharmacology, so I examined whether cholinergic and $GABA_A$ergic afferent depolarizations occur through separate mechanisms. To this end, I showed that d-tubocurarine does not affect muscimol induced depolarization of afferents. Additionally, I showed that picrotoxin, which *does* blocks the muscimol induced depolarization, has no effect on afferent depolarization produced by ACh and neostigmine or epibatidine further, demonstrating distinct mechanisms of action for GABAergic and cholinergic depolarization of primary afferents. Finally, I showed that collapsing the chloride gradient with bumetanide nearly blocked the ability of muscimol, but not ACh and neostigmine to depolarize primary afferents.

In conclusion, DC depolarization of afferents is mediated by activation of and cation influx through α3, α6, and/or α9-containing nAChRs located directly along afferent axons. This depolarization is not due to overlapping actions at $GABA_ARs$. Therefore the substrate exists for a cholinergic depolarization of afferents contributing to PAD.

4.5.3 Significance of depolarization of primary afferents via nAChR activation

It has been demonstrated that GABA is able to produce PAD in primary afferents (reviewed in (Rudomin, Romo et al. 1998) resulting in PSI of afferent transmission onto postsynaptic spinal targets. This study examines the role of ACh acting at nAChRs ability to produce PAD that is independent of GABA and $GABA_ARs$. Why do two separate mechanisms producing PAD exist?

4.5.3.1 Reversal potential implies nAChRs are involved in spike generation in afferents.

The reversal potential for GABA in primary afferents is -26 mV in primary afferent neurons (Gallagher, Higashi et al. 1978), while nAChRs have a reversal potential near 0 mV (Harnaen, Na et al. 2007). With a larger driving force and a great potential for depolarization, it is tempting to speculate that the activation of nAChRs on primary afferents more readily leads to action potential generation. Additionally, GABA has been shown to have less of a depolarizing effect on C-fibers (Desarmenien, Santangelo et al. 1984), which may allow for nAChRs to exert a greater influence on the excitability of these afferents. All of the nicotinic receptors that were examined appeared on small diameter DRG neurons, supporting their role in controlling C-fiber excitability. I have shown the ability of ACh and nAChR agonists to lead to increased spiking in the dorsal roots – both in continuity with the spinal cord and isolated from it – as well as anatomical evidence for the presence of nAChRs in peripheral nerves. It seems then that these receptors can produce spiking that travels both ortho- and anti-dromically.

4.5.3.2 Antidromic spiking and inflammation

Neurogenic inflammation is the inflammatory response mediated by chemicals released from sensory nerve endings. It has been shown that afferent depolarization is increased during inflammation and the increase in the depolarization state of afferents is accompanied by increased DRRs and dorsal root spiking (reviewed in (Willis 1999)). I have shown that activation of nAChRs along the length of the axon lead to both PAD and increased spiking in primary afferents and that dorsal root reflexes can be blocked by nicotinic antagonists. And it has been demonstrated that activation of α7 nAChRs can lead to LTP in synapses from primary afferents to dorsal horn interneurons (Genzen and McGehee 2003). Increased spiking in afferents exposed to cholinergic agonists seems to contrast with reports of the analgesic actions of nicotine and epibatidine (Lawand, Lu et al. 1999). However, in a recent study (McIntosh, Absalom et al. 2009), it has been demonstrated that block of α9-containing nAChRs is able to increase

nociceptive threshold, and it is at these receptors, that both nicotine and epibatidine act as antagonists (Verbitsky, Rothlin et al. 2000). These studies fundamentally alter the current understanding of how sensory information is integrated in the spinal cord. I have shown that nAChRs play an integral role in production of a significant portion of the DRP, transmission from a select population of primary afferents onto spinal interneurons and production of DRRs in primary afferents. The results presented above also provide support for a role for nAChRs in regulation of afferent excitability and signaling in inflammatory states and provide a framework for the development of novel therapeutics aimed at more specific control of primary afferent transmission onto both central and peripheral targets. Thus, manipulation of nicotinic acetylcholine receptors has the ability to modulate both neuropathic pain and neurogenic inflammation paving the way for development of appropriately selective drugs to target nAChRs.

4.5.3.3 Analysis, Significance and Statistical Power

Much of the analysis performed for this chapter resulted in significant changes from control events in the presence of nicotinic effectors, as noted in the preceding sections. It is important to note that in several cases trends in the data, seen as non-significant changes from control values, did not reach significance due to large variability in the data or small sample sizes in individual groups. In such cases, the statistical power of the analysis was too low to conclude that there was no change from control. I have noted this in the text when relevant.

Table 10 Summary of nicotinic antagonist actions on evoked and afferent depolarizations.

		α-bungarotoxin α7, α9 and α10	**d-tubocurarine** α1, α2, α3, α4, α6, α7, α9, and α10	**DHβE** α2, α3, and α4	**Mecamylamine** α3, α6 and α9	**MLA** α7
DRP	*A-fiber component*		↓(DR, cut)			No change
	C-fiber component		↓(DR, cut)		↓ ns	No change
	unseparated	↓	↓	No change	↓ (area, suggesting C-fiber)	↓ on wash
DRRs		↓	↓	No change	↓	No change
Spontaneous DRPs		↓	↓	No change	No change	No change
EFP	*A1*	No change	No change (DR, cut)			
	A2	↓ ns	↓ (cut)			
	C		↓ ns (DR)			
DR spiking		↓ (50% of experiments)	↓	No change		↓ (50% of experiments)
AV		No change	No change	No change	No change	No change
ACh/neo DC depolarization			↓		↓	
ACh/neo DC depolarization with TTX or in isolated root			↓	No change	↓	

↓ = decrease or block of a given signal. ↑ = increase of a given signal. DR = the signal resulting from dorsal root stimulation, cut = the signal resulting from cutaneous nerve stimulation. When fields are blank, that signal was not tested for that particular drug. ns = not significant, but with a trend in the direction of the arrow.

Table 11 Summary of nicotinic agonist actions on evoked and direct actions leading to PAD

		Acetylcholine	**Neostigmine** (cholinesterase inhibitor)/**ACh**	**Nicotine** nicotinic agonist; antagonist at α9, and α10	**Epibatidine** nicotinic agonist; antagonist at α9, and α10	**PNU** α7 positive allosteric modulator
DRP	*A-fiber component*		↓ (cut)		↓ ns (DR, cut)	No change (cut)
	C-fiber component		↓ ns (cut)		↓ ns (DR, cut)	No change (cut)
	unseparated	↓	↓ (DR)	↓	↓ (DR, cut)	
Spontaneous DRPs		↓	↓	↓	↓	
EFP	*A1*		↓ (DR, cut)		↓ ns	
	A2	↓	↓(DR, cut)		↓ ns	
	C	↓	↓ (DR)			
DR spiking		↑	↑	↑	↑	↑
AV		↓	↓		No change	No change
DC depolarization		YES	YES	YES	YES	YES
DC depolarization with TTX		YES	YES	YES		

↓ = decrease or block of a given signal. ↑ = increase of a given signal. DR = the signal resulting from dorsal root stimulation, cut = the signal resulting from cutaneous nerve stimulation. When fields are blank, that signal was not tested for that particular drug. ns = not significant, but with a trend in the direction of the arrow.

Chapter 5 General Discussion and Summary

5.1 Summary and Discussion of Key Findings

In the first study, I examined the unusual nature of the pharmacology of primary afferent evoked PSI resulting from afferent stimulation. While PSI of primary afferents is traditionally assumed to be a trisynaptic circuit with a first-order glutamatergic interneuron and a last-order GABAergic interneuron that synapses onto primary afferent terminals (Figure 6A), the pharmacology presented here suggests that the mechanism is much more complex. I combine previously published studies with my own work examining the complex nature of afferent-evoked PAD to demonstrate that PAD can persist in the presence of some GluR antagonists and is depressed by nicotinic AChR antagonists. Furthermore, I present evidence of direct release of ACh from primary afferents, which could result in afferent to afferent PAD mediated in part by nAChRs. Taken together these results lead to the conclusion that PAD may be at least partly independent of last order GABAergic interneurons and classical $GABA_A$ receptors.

A closer examination of the actions of GluR antagonists show that block of GluRs (AMPA/kainate and NMDA) with kynurenate depress the late portion of the DRP, but the early portion is unaffected, even though ventral root reflexes are abolished. A combination of the NMDAR antagonist, APV, and the non-NMDAR antagonist, NBQX, block both the DRP and VRP. However, a combination of kynurenate, APV (NMDAR antagonist) and GYKI52466 (non-NMDAR glutamatergic antagonist) completely abolish the ventral root response, but the DRP shows only a slight depression until higher concentrations of GYKI52466 are used; these higher concentrations lead to complete block of the DRP. Interestingly, the quinoxaline GluR antagonists (CNQX, DNQX, and NBQX – all used as non-NMDAR GluR antagonists) show non-specific actions at both glycine (Meier and Schmieden 2003) and $GABA_A$Rs (Jarolimek and Misgeld 1991). The differential sensitivity of glutamate receptor antagonists to afferent-evoked

DRPs vs. reflex actions has been observed previously *in vivo* (Farkas and Ono 1995). In sum, these variable results with the glutamate receptor antagonists suggest that a component of PAD may not require glutamatergic synaptic transmission.

If afferent-evoked PAD is partially independent of glutamatergic transmission, then another transmitter must be released from primary afferents that leads to PAD. Other candidate transmitters for production of the DRP include taurine and β-alanine, which both activate $GABA_A$ receptors to produce PAD (Nicoll and Alger 1979). Microdialysis experiments demonstrate taurine and β-alanine release comparable to glutamate release following activation of primary afferents (Paleckova, Palecek et al. 1992). There is also evidence of taurine immunoreactive primary afferents (Lee, Renno et al. 1992). I also provide evidence that taurine and β-alanine could be released from primary afferents and act on $GABA_A$Rs at their terminals to provide a direct mechanism of PSI. Both of these transmitters are localized to DRG neurons, and taurine co-labels with vGluT1 (a marker for primary afferent terminals) in the dorsal horn of the spinal cord. These transmitters, however, likely represent only a minor contribution to afferent-evoked PAD, as application of them only has a minor effect on the DRP.

Another candidate transmitter that appears to play a more significant role in afferent evoked PAD is ACh. To address this, I show that many primary afferents are able to synthesize, package and degrade ACh, making it highly likely that they also release ACh. In addition, I provide preliminary evidence that nAChR antagonists can depress the DRP. In Chapter 4, I examine cholinergic contribution to PAD in great detail. These findings suggest PAD mechanisms may be much more diverse than previously imagined, conceivably separated into distinct mechanisms for each genetically distinct afferent fiber population.

After demonstrating that PAD may occur though mechanisms partially independent of glutamatergic transmission, combined with the evidence for direct release of PAD producing

transmitters from primary afferents, it was imperative to examine how much of PAD could be produced independent of the dogmatic trisynaptic pathway consisting of a primary afferent synapsing onto a glutamatergic interneuron followed by a last order GABAergic interneuron with an axoaxonic synapse onto primary afferent terminals. The data presented in subsequent study described in Chapter 3 challenge the assumption, based exclusively on latency measures, that PAD predominantly involves a minimally trisynaptic pathway involving excitatory and GABAergic interneurons (Eccles, Magni et al. 1962; Jankowska, McCrea et al. 1981; Rudomin and Schmidt 1999). I show that the DRP not only remains but is unaffected after isolation of monosynaptic actions, either pharmacologically with mephenesin or by using a high divalent cation-containing aCSF. This is consistent with an earlier *in vivo* report on the actions of mephenesin (Farkas, Tarnawa et al. 1989). Evidence of monosynaptic isolation was corroborated with intracellular recordings in dorsal horn neurons that showed block of longer latency excitatory currents. Even after near-complete block of synaptic transmission with a high Mg^{2+}/low Ca^{2+} aCSF, a portion of the DRP remained, providing further support for a more direct form of PAD.

The most conservative interpretation of these data is that PAD is mediated by disynaptic pathways with axo-axonic synapses onto primary afferents. PAD may also be produced by disynaptic dendroaxonic pathways. In turtle, a non-spiking microcircuit generates a DRP, evoked following stimulation of high-threshold TTX-insensitive fibers (Russo et al., 2000). Primary afferents contain numerous dendroaxonic synapses (Alvarez 1998), so it is conceivable that microcircuits based on these synapses produce PAD at various low-threshold afferents. PAD produced by dendroaxonic actions would be resistant to mephenesin and high divalent cation solutions. This would also be consistent with DRPs seen under conditions of minimal transmission where post-synaptic spiking would not be required.

Finally, direct negative feedback could occur, as supported by a portion of the DRP remaining after block of synaptic transmission. If more DRP production involves more direct actions, how might it be mediated? Direct feedback would require that primary afferents release transmitter(s) acting on $GABA_A$ or $GABA_A$-like receptors, but numerous labeling studies have failed to support GABA as a transmitter localized in primary afferents (Todd, 1996).

As alluded to above, another distinct possibility is that ACh, with a possible contribution from taurine and/or β-alanine, is co-released from primary afferents to act on bicuculline-sensitive nicotinic receptors. Peripheral choline acetyltransferase (pChAT) is found in both small- and large-diameter primary afferents (Bellier and Kimura 2007) and immunolabeling studies identify pChAT in myelinated dorsal column primary afferents (Yasuhara, Aimi et al. 2008). Large-diameter afferents preferentially express the vesicular ACh transporter as well as acetylcholinesterase (Willis and Coggeshall 1991; Tata, De Stefano et al. 2004; Bellier and Kimura 2007). Primary afferents also contain bicuculline-sensitive α9 and α10 nicotinic ACh receptor subunits (Rothlin, Katz et al. 1999; Lips, Pfeil et al. 2002; Alexander, Mathie et al. 2007). In the previous chapter, I showed that nicotinic receptor antagonists with reported actions on α9 receptors (Hochman, Shreckengost et al. 2010) are capable of depressing the DRP. Figure 14B summarizes these possibilities. Chapter 4 expands on the role of nAChRs in PAD producing pathways, and further examines the possibility of distinct actions of ACh on genetically and anatomically identified afferent populations.

5.3 Nicotinic Involvement in Primary Afferent Depolarization

The final study presented here examined nicotinic involvement in PAD and discovered subunit-specific nAChR antagonists on components of the DRP and on direct actions of nAChRs on primary afferent terminals. Several nicotinic antagonists (d-tubocurarine, α-bungarotoxin and mecamylamine) are able to depress the DRP. These drugs act jointly at α9-containing nAChRs.

Only the nicotinic antagonists that act at α9 nAChRs have an effect on the DRP and accompanying A-fiber mediated dorsal root reflexes (DRRs). Block of nAChRs depressed the DRP evoked from both dorsal root and cutaneous afferent stimulation. When stimulating cutaneous afferents, I was able to distinguish between A- and C-fiber mediated components of the DRP. Both components were similarly depressed by d-tubocurarine and mecamylamine, though mecamylamine did show a trend toward a preferential depression of the C-fiber component of the DRP. Interestingly, immunohistochemistry suggests differential nAChR distribution in primary afferent populations. Both α6 and α9 nAChR subunits appear preferentially in small diameter cells, which would correspond to involvement in the C-fiber component of the DRP. On the other hand α3 and α7 nAChR subunits are found on medium to large diameter cells, which would support a role in contributions to the A-fiber DRP. Depression of the A-fiber DRP is likely mediated through depression of transmission of slower conducting A-fibers, as evidenced by a concurrent depression in the second component of the monosynaptic A-fiber mediated EFP in the presence of d-tubocurarine and α-bungarotoxin, as discussed below. Unsurprisingly, pharmacological results do not support an α7 contribution to the DRP, probably due to this receptor's high desensitization rate (Giniatullin, Nistri et al. 2005) making it unlikely to contribute to a long-lasting phenomenon like the DRP. It is possible that specific actions on A- and C-fiber mediated portions of the DRP were not observed, due to the broad spectrum antagonism d-tubocurarine at nearly all nAChRs.

EFPs were concurrently monitored in the deep dorsal horn at the depth where the largest signals were elicited by cutaneous afferent stimulation at low intensities. EFPs are a measure of monosynaptic transmission from primarily A-fiber primary afferents onto spinal interneurons. Both d-tubocurarine and α-bungarotoxin caused a depression of EFPs. A depression of monosynaptic field potentials indicates a reduction in synaptic transmission to interneurons in the vicinity of the intraspinal electrode. While measuring EFPs cannot distinguish between pre- or

post-synaptic mechanisms, it does demonstrate a depression of transmission at the first synapse from primary afferents entering the spinal cord. This depression of transmission implies the presence of nAChRs located either on primary afferent terminals or on spinal interneurons (Figure 37). Previous studies have demonstrated a case for depression of EFPs being the result of PAD (Riddell, Huber, 1995).

In summary α9 nAChRs are involved in production of the DRP and A-fiber DRRs, likely through transmission from slower conducting cutaneous A-fibers (likely Aδ). α7 nAChRs are, at least partially involved in generation of spontaneous spiking activity in the dorsal roots, and potentiation of these receptors can increase spiking activity in both the dorsal root and cutaneous afferents in the periphery. Taken together, these data suggest selective actions of nicotinic receptor subtypes on specific afferent classes.

Next the effects of ACh and nicotinic agonists on the DRP and EFP were examined. ACh, nicotine, and epibatidine also caused a significant decrease in DRP amplitude and area. In the presence of ACh, both the A- and C-fiber components showed an observable depression of the DRP. Epibatidine showed no difference in A- and C-fiber components of the DRP. Co-application of ACh and the cholinesterase inhibitor, neostigmine, caused a decrease in DRP that was much larger than that seen by agonists alone. This discrepancy is probably largely due to a) block of ACh hydrolysis at the synapse and b) the increased desensitization rates of nearly all nAChRs in the presence of nicotine (Chavez-Noriega, Gillespie et al. 2000) and epibatidine (Genzen, Van Cleve et al. 2001). The immediacy of hydrolysis of ACh is the reason current decay begins quickly at cholinergic synapses (Giniatullin, Nistri et al. 2005). Though both nAChR agonists and antagonists have a similar effect on DRP amplitude and area, the DRP reduction in response to agonists is likely due to afferent depolarization. When evoked, the DRPs are reduced in size, as the afferents in their depolarized state are closer to the reversal potential of the DRP. This depolarization is seen in DC recordings of the polarization state of primary

afferent axons and is evidenced by an increase in spontaneous firing in the dorsal root, suggestive of fiber depolarization.

ACh and neostigmine in combination decreased both dorsal root and cutaneous evoked EFPs. This is likely due to depolarization block of afferent transmission. Support for this is seen by the ability of ACh and nAChR agonists to lead to a direct depolarization of primary afferents, as discussed below.

In summary, ACh and nicotinic agonists lead to a depression of afferent evoked DRPs and EFPs though a DC depolarization of afferents, which is mediated by activation of, and cation influx through, α3, α6, and/or α9-containing nAChRs located directly on afferent axons.

5.4 Roles of Nicotinic Presynaptic Inhibition versus Facilitation

While PAD has long been thought to underlie inhibition of sensory transmission in the spinal cord, both the reversal potential of nAChRs and the nature of α7 and α9-containing nAChRs lead to questions of whether nicotinic mediated PAD could actually be facilitative at primary afferent synapses. Both α7 and α9-containing nAChRs are largely permeable to Ca^{2+} (Hefti and Smith 2000; Gong, Zhou et al. 2003), and an increase in Ca^{2+} at the synapse could very well facilitate transmission through Ca^{2+} accumulation. In addition, α7 nAChRs are rapidly desensitizing, and while in the absence of modulators they contribute little to DRP production and PAD, even small increases in intracellular calcium at a presynaptic terminal could still play a large role in facilitation of transmission.

In other areas of the nervous system, nAChRs underlie presynaptic facilitation of release of GABA (Luo and Dessem 1999), glutamate (McGehee, Heath et al. 1995), norepinephrine, and 5-HT (Hellstrom 1998). Even in spinal neurons in the dorsal horn, activation of α7 nAChRs and NMDARs on primary afferents mediates both short- and long-term plasticity (Genzen and McGehee 2003). I have shown an increase in spiking activity in primary afferents in the presence

of nicotinic agonists. These spikes likely have both peripheral and central actions. I have shown that DRRs are sensitive to some nicotinic agonists, and it has previously been reported that DRRs can trigger large post-synaptic responses (Eccles, Schmidt et al. 1962), such that production of DRRs might be directly linked to afferent transmission. It remains to be elucidated which nAChRs are involved in presynaptic inhibition versus facilitation and in which distinct afferent populations these opposing mechanisms are active.

5.5 Implications for Therapeutic Pharmacological Control of Sensory Integration

After spinal cord injury, the sensory system becomes hyper-responsive, and a primary strategy for therapeutic control is to amplify presynaptic inhibitory actions. There is evidence for plasticity of PSI after SCI (Hultborn and Malmsten 1983; Tillakaratne, Mouria et al. 2000; Tillakaratne, de Leon et al. 2002; Dougherty 2005), though the role of nAChRs in PAD after injury remains to be examined.

Understanding the mechanisms limiting sensory transmission at the level of the spinal cord is critical to discovering novel treatments for sensory dysfunction. For example, spasticity is common problem after stroke, cerebral palsy, multiple sclerosis and spinal cord injury, and much of this afferent over-activity is due to loss of descending inhibitory control systems. There are three primary medications used to treat spasticity: baclofen, diazepam, and dantrolene. All have debilitating side effects and still do not eliminate spasticity entirely. Baclofen acts by decreasing glutamate release on afferents by activation of $GABA_B$ and GABA release on GABAergic terminals of interneurons by activation $GABA_B$ autoreceptors. Diazepam potentiates $GABA_A$ receptors, and like baclofen, serves to increase the inhibitory tone of the nervous system. Dantrolene is a Ca^{2+} channel blocker, and as such, has widespread CNS actions. Cholinergic involvement in PSI is very attractive as a potential therapeutic target, as α9/α10-containingnAChRs have an extremely limited distribution in the CNS and are only found on afferents in the spinal cord, pituitary pars tuberalis, the olfactory epithelium, and the sensory hair cells of the cochlea (Elgoyhen, Johnson et al. 1994). Hence, the possibly of selectively modulating sensory transmission at primary afferent synapses with limited nonspecific drug actions is quite appealing clinically.

Nicotine and drugs that target nicotinic receptors are already becoming attractive therapeutic models for treatment of neuropathic pain (Matsumoto, Xie et al. 2007; McIntosh, Absalom et al.

2009). However, receptor desensitization and differential actions of nicotinic agonists on various nAChRs (e.g. nicotine's antagonism of α9 nAChRs) necessitate greater understanding of receptor distribution and action on regulation of sensory information (Kogure, Ishizaki et al. 1999). Expression of nAChRs along sensory axons provides even greater potential for novel drug targets, as the compounds do not need to cross the blood brain barrier to have an effect. Subcutaneous and intramuscular injection of α9/α10 nAChRs antagonist is able to acutely alleviate traumatic, inflammatory, and metabolic neuronal injury (McIntosh, Absalom et al. 2009). Today, nAChRs are also being recognized as players in more diverse peripheral phenomena, such as leukocyte proliferation (Asato, Butler et al. 2000) and release of norepinephrine from adrenal chromaffin cells (Sandall, Satkunanathan et al. 2003), opening the door for development of drug treatments targeting these peripherally located nicotinic receptors.

5.7 Future Directions

Future studies must distinguish whether antidromic spiking and PAD are affected by separate mechanisms, which can be accomplished by intra-axonal recordings from primary afferents. In this way, one can definitively observe spiking in the absence of PAD and then relate such spiking to nAChR activation.

Determining when pre- versus post-synaptic mechanisms of action lead to a reduction in EFPs will also be critical in future studies. Such differentiation can be accomplished via paired-pulse recording protocols, which distinguish between pre- and post-synaptic mechanisms by examining the ratio of the amplitude of the first response to the amplitude of the second response. A change in ratio in the presence of a nAChR antagonist suggests a presynaptic site of action (Gaillard, Bonfield et al. 2008).

Finally, the data herein demonstrate actions of specific nAChRs on subclasses of primary afferents. A larger data set using subunit specific antagonists to examine A- and C-fiber evoked

effects is critical. These data could be confirmed and expanded upon with the use of transgenic animals lacking specific nAChR subunits, or through examining drug actions due to activation of molecularly identified afferent populations through transgenic mice expressing channel rhodopsin in genetically specified afferent populations. Together these studies would build a platform for developing therapeutic agents for controlling aberrant afferent signaling in disease or injury states, with the specificity of nicotinic subunit expression on afferent subpopulations limiting potential unwanted side effect of pharmacological intervention.

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