An Investigation of the Role of the Intraspinal Cholinergic System in the Modulation of Motoneuron Voltage Threshold

By

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba

in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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Winnipeg, Manitoba, Canada

Abstract

Previous work has demonstrated that rhythmic motor outputs, such as fictive locomotion and scratch induce a hyperpolarization of the voltage threshold (V_{th}) for action potential initiation in spinal motoneurons which would increase motoneuron excitability. Descending monoaminergic mechanisms were implicated in mediating this effect; however, the recent observation that this change in V_{th} can persist during fictive scratch in adult cats following acute transection of the cervical cord revealed that modulatory systems intrinsic to the spinal cord also have the ability to regulate motoneuron excitability during motor behaviour. The neuromodulatory identity of this intraspinal system has yet to be determined. This thesis addresses: 1) whether acetylcholine (ACh) is able to modulate spinal motoneuron V_{th}, and 2) whether endogenous ACh modulates motoneuron excitability during motor activity occurring without intact descending modulatory systems.

Our first study (Paper 1) investigates whether the ACh from exogenous and/or endogenous sources alters motoneuron V_{th}. In this study we made intracellular recordings of lumbar motoneurons from neonatal rats to pharmacologically manipulate muscarinic and nicotinic receptor activity. The results show that cholinergic inputs can induce either V_{th} hyperpolarization, V_{th} depolarization or no change in V_{th} depending on the activity state of the network, the ACh concentration, and influences from other systems.

Our second study (Paper 2) investigates whether an intraspinal cholinergic system induces V_{th} hyperpolarization during rhythmic motor output when descending modulatory systems are disrupted. For this study we developed an *in vitro* neonatal rat spinal cord preparation to elicit rhythmic motor activity independently of brainstem or lumbar cord

stimulation. Intracellular recordings from motoneurons allowed comparison of the V_{th} prior to and during rhythmic motor output, both in the absence and presence of cholinergic receptor antagonists in the lumbar cord. The results show that intraspinal cholinergic mechanisms are active and importantly contribute to modulation of motoneuron V_{th} during motor output.

We suggest that in addition to descending modulatory inputs, the spinal cholinergic system regulates motoneuron V_{th} to either facilitate or inhibit recruitment according to the state of the motor network. Modulation of motoneuron excitability by modification of distinct membrane properties and as a result of the activation of different modulatory systems during diverse motor behaviours is discussed.

This work is the first to demonstrate the role of cholinergic mechanisms in regulating motoneuron excitability through modulation of V_{th} in an activity based context, and therefore outlines a spinal modulatory system that would contribute to motor control in both normal and pathological states.

Acknowledgements

First of all, I would like to thank Dr. Brent Fedirchuk, who received me in his lab with openness and cordiality, despite the fact that I was part of the threatening Mexican invasion. Thanks for giving me the opportunity to accomplish one of my biggest dreams in life! You gave me the right amount of the freedom and insightful guidance I needed. Your approachability, generosity, and support were essential for my formation as a researcher. Your great sense of humor taught me to relax a little bit and take things with less apprehension, a huge lesson for me given my skittish personality!

Thanks to my committee members – Drs. Dave McCrea, Mark Fry, Kristine Cowley and Larry Jordan, for your guidance, input, support, and for challenging me with new ideas. You were a constant source of encouragement and inspiration throughout my training. I feel very fortunate for being part of this outstanding research group.

To my fellow graduate students – Natasha, Nicole, and Emma. Thanks for your friendly support and help. You made me feel at home in this country. Special thanks to my close friend and fellow graduate student, Erika Couto. We started this adventure together, from Mexico to Canada, and your company highly enriched all my experience. I truly enjoyed every talk we held, from scientific to philosophical. I may have a PhD now while you ended with an MSc, but I admire you as a senior leader in the fields of motherhood and personal growth!

I owe lots of gratitude to Dr. Kevin Power, who taught me a great deal in handling the lab secrets and keeping me motivated with pep talks and fun memories. You were a role model of the mentor I want to become! Thank you! and... See? I graduated *before* you actually retired! Yaaay!

Also, special thanks to Dr. Katinka Stecina for your critical input and most importantly for your friendship and generosity. All of that has been very important and necessary at this stage of my life.

To Matt Ellis, Gilles Detillieux, Maria Setterborn, and Sharon McCartney, thanks for your valuable and very much appreciated technical assistance and also for your friendship. Thank you to Gail McIndless and Judy Olfert, for sharing your administrative expertise and for all your support.

Thank you to the Canadian Institutes for Health Research and the Manitoba Spinal Cord Injury Research Committee for providing financial support for this research.

Dedication

I dedicate this thesis to my family.

To my parents for their deep, unconditional and endless love. Without your constant support I simply wouldn't be able to successfully accomplish this dream of mine. You were always there for me, from taking care of my child while I was at the lab, to encouraging me when my morale was not as determined as it needed to be. Mom, your insights about the mysterious side of life have always complemented my scientific view on all things, thank you for sharing your wisdom and using your incredible healing power with me! Dad, as for your students, you are my role model of the intelligent, honorable and kind person I want to be. You are my hero! Thank you both for trusting in me, keeping me strong and focused in what is most important in life. I love you!

To my husband, Cesar, who was my companion in this bumpy road towards discovering ourselves through our respective professional development. This journey was as difficult for you as it was for me, and we were not always in the best shape to provide support to each other. However, we have both gained a great deal of personal growth in the process. Thank you for overcoming your impatience and staying by my side. This thesis is for you. I told you I would finish it!

To the memory of my sister, Alma Luz. You left this dimension middle way throughout my doctoral program, leaving behind the two biggest lessons I've learned in life: to allow myself and others enjoy life with freedom, and to love people without judgement, fully accepting them with all their good and not so good personality features. I wish I could let you know I finally understood this thanks to you.

To my son, Amilkar. You were the trigger of a transformation inside me I believed impossible. Thank you! Embrace all, small and big challenges with confidence: you can achieve anything you set your mind to. And don't forget to enjoy the journey towards attaining your dreams! I love you profoundly.

List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AHP	afterhyperpolarization
AIS	axon initial segment
AnkG	ankyrin G
C1-C2	cervical spinal segments 1-2
Ca^{2+}	calcium
ChAT	choline-acetyl-transferase
CNS	central nervous system
CPG	central pattern generator
DA	dopamine
f/I	frequency/current
GABA	gamma-aminobutyric acid
GRPR	gastrin releasing peptide receptor
GRP_{18-27}	gastrin releasing peptide receptor agonist 18-27
Hz	hertz
K^+	potassium
L1-L5	lumbar spinal segments 1-5
MLR	mesencephalic locomotor region
ms	miliseconds
mV	millivolts
NA	noradrenaline
nA	nanoamperes
Na^+	sodium
NMDA	N-methyl-D-aspartate
NO	nitric oxide
PIC	persistent inward current
PKC	protein kinase C
R_{in}	input resistance
s	seconds
SD	somatodendritic
SP	substance P
STG	somatogastric ganglion
VAChT	vesicular acetylcholine transporter
VLF	ventrolateral funiculus
V_m	membrane potential
VR	ventral root
V_{th}	voltage threshold
5-HT	serotonin

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Chapter I.

GENERAL INTRODUCTION

Movement, the basic component of behaviour, is produced by a complex circuitry within the central nervous system (CNS) that ultimately converges onto projection neurons that directly activate muscle fibers. Alpha motoneurons are these output neurons recognized by Sherrington (1906a) as the "final common path" that transforms neural activity into muscular contraction. Understanding how CNS internal actions translate into motor behaviour therefore requires recognition of the mechanisms that determine motoneuron firing. Research on the activity and connectivity of spinal motoneurons, the first neurons recorded intracellularly (Brock et al, 1952; 1953), has revealed several fundamental properties of excitable cells and synaptic transmission that apply to most neuron types (see reviews by Renkling et al, 2000; Powers & Binder, 2001; Fyffe, 2001).

Initial studies on quiescent motoneurons regarded these cells as the sites where excitatory and inhibitory inputs simply summed until excitation was enough to activate the motoneuron (see Powers & Binder, 2001 for review). Subsequent observation of motoneuron activity in the context of actual motor behaviour revealed a dynamic responsiveness of motoneurons to their inputs. It is now well known that both passive and "active" properties of the motoneuron membrane account for the complex integration process that defines motoneuron firing during movement (reviewed in Heckman et al, 2009). Because neuromodulators control changes in these motoneuron properties and regulate almost every aspect of motor network functioning, they have a strong impact on motor behaviour. A clear understanding of the mechanisms involved in the production of movement, therefore, requires

comprehension of how neuromodulatory systems influence motoneuron and motor network behaviour and vice versa.

The General Introduction of this thesis reviews some of the evidence that modulatory systems control motoneuron excitability, focusing particularly on the membrane potential for action potential initiation, referred to as the voltage threshold (V_{th}). This is followed by two manuscripts describing the work and a General Discussion of the putative interaction between V_{th} and network state.

Central Pattern Generators

Historical background

Since the late 19th century it has been recognized that rhythmic movements such as locomotion could be elicited in animals without intact supraspinal structures. Sherrington's (1906b, 1910) seminal work in spinal dogs and cats led him to suggest that complex movements, including locomotion and scratch, resulted from chains of reflex actions from sensory afferents to spinal centers. Opposing this view, Brown (1911) demonstrated that networks intrinsic to the spinal cord had the capability to generate locomotor-like activity without afferent feedback. The spinal network able to generate stepping independently of descending and sensory inputs is now commonly referred to as central pattern generator (CPG) for locomotion (Grillner, 1981).

Brown (1914) proposed a "half-center" model of the locomotor CPG structure consisting of two groups of spinal neurons controlling flexor and extensor motoneuron pools. Each half center was organized to produce rhythmic activity and to reciprocally inhibit each other. Activity in one group (e.g. extensors) then would simultaneously inhibit the excitation

of the antagonist group (flexors), and after a refractory period, the opposite group became dominant (flexor activity). Alternation between flexor and extensor groups would cycle repeatedly, ultimately producing locomotion. To date, a large variety of models have been proposed regarding the organization of the locomotor CPG. The most recent proposed CPG architectures incorporate separate circuitries for rhythm generation and pattern formation, as well as interneuronal components receiving sensory input (i.e., two-and three-level CPGs) that all together explain complex features of locomotion such as mixed-synergy motor patterns, regulation of locomotor speed, stumbling corrections, and deletions (reviewed in McCrea & Rybak, 2008).

Mammalian models for the study of CPG output

The term CPG is used to refer to neuronal networks controlling rhythmic, stereotyped and well organized motor behaviours, such as swimming, flying and peristalsis in invertebrates and breathing, swimming, chewing, scratching and stepping in vertebrates. In mammals, the study of locomotion has received the most attention and cats and rodents were used as primary models. To investigate the modulation of motoneuron excitability associated with rhythmic motor activity, we have employed the *in vitro* neonatal rat spinal cord preparation using the *in vivo* adult cat preparation as a reference. The following sections provide a brief overview of these two preparations.

The *in vivo* cat preparation

The *in vivo* cat provides an acute model of adult, fully-developed motor system. Although this preparation allows for several modifications, most studies examining the

behaviour of individual neurons during motor activation have used decerebrated cats in which the brain above the level of the midbrain is surgically removed leaving an intact brainstem and spinal cord. Removal of pain processing portions of the brain allows discontinuation of anesthesia so that the remaining CNS structures can be activated with electrical stimulation. Dorsal laminectomy at particular segments exposes the spinal cord and hindlimb nerve dissection is performed to record motor activity. Because a muscular paralyzing agent is applied to allow stable intracellular recordings, the term "fictive" is used to refer to motor patterns that would drive movement during intact muscle activation conditions. The main limitation of this preparation is the difficulty of modifying the extracellular fluid surrounding spinal neurons, which complicates the assessment of their pharmacological properties. Focal iontophoresis has been used successfully in some studies, although the use of such technique in an *in vivo* preparation is quite challenging.

Cat locomotion

Locomotion in the cat, as described by kinematic studies (see for example Abraham & Loeb, 1985), consists of two general phases of activity, swing (i.e. flexion) and stance (i.e. extension). During swing, flexion of all joints moves the limb upward and forward. Extension starts first in the ankle and knee while the hip is still flexed. After the foot touches the ground, the ankle and knee joints flex passively and change angle while the hip moves forward over the foot. Extension of all joints finally propels the body forward. The muscle (and nerve) activity pattern of flexors during swing and extensors during stance has been characterized with detail (Grillner, 1981). The features of muscular activation (e.g. timing, pattern, burst

duration and amplitude) vary in relation to the speed, inclination and direction of locomotion (Rossignol, 1996).

Eliciting fictive locomotion in cats

Supraspinal structures that activate the spinal CPG to produce locomotion have been the subject of intensive research in the last 70 years (see reviews by Grillner, 1981; Armstrong, 1986; Jordan, 1991, 1998; Whelan, 1996; Grillner et al, 1997; Mori et al, 2001; Le Ray et al, 2011). Typically, electrical stimulation of a functionally defined area in the brainstem, the mesencephalic locomotor region (MLR) is used to induce locomotion (Shik et al, 1966). Projections from the MLR contact reticulospinal neurons in the lower brainstem which in turn project to the spinal cord through the ventrolateral funiculus (VLF) to activate the locomotor CPG. Descending inputs via the VLF are considered essential for the initiation of locomotion in cats, as lesion of ventral pathways of the spinal cord abolishes MLR-evoked locomotion (Afelt, 1974; Steeves & Jordan, 1980; Eidelberg et al, 1981; Shefchyk et al, 1984; Noga et al, 1991).

Pharmacological agents can also induce fictive locomotion in decerebrate cats. Initial experiments showed that intravenous administration of the catecholaminergic precursor L-DOPA uncovers reflexes with a pattern of nerve activation consistent with locomotion (Jankowska et al, 1967a,b). Subsequent studies (reviewed in Rossignol 1996; Rossignol et al, 1998, 2011; Jordan et al, 2008) have revealed the involvement of alpha-2 noradrenergic (α2 NA) and N-methyl-D-aspartate (NMDA) glutamatergic agonists in the production of cat locomotion. Chemical activation of the MLR with agonists of gamma-aminobutyric acid (GABA) also induces locomotion in decerebrate preparations (Garcia-Rill et al, 1985).

Although the serotonergic (5-HT), dopaminergic (DA) and cholinergic (ACh) systems have been implicated in pathways initiating locomotion, their agonists have failed to induce rhythmic activity in spinal cats.

Cat scratch

Scratching is a rhythmic hindlimb motor behaviour different from locomotion. Initially described by Sherrington (1906a,b, 1910, 1917), scratch was recognized as a stereotypic response to cutaneous stimuli located around the ear, head or neck. In intact cats, the scratch pattern consists of tonic flexion of the hindlimb ipsilateral to the stimuli site for positioning (approach phase) followed by flexion and extension movements of all joints, alternating at a frequency of 5-7 Hz (rhythmic phase) (Abraham & Loeb, 1985, Kutha & Smith, 1990). The contralateral limb may remain inactive or show tonic extensor activity to support the body (weight support).

Most features of the real scratch pattern are conserved in the fictive reflex, except a longer duration of flexor activity in the latter. Fictive scratch bouts have a 250 ms cycle duration with flexor and extensor phases of 200 ms and 50 ms, respectively (Berkinblit et al, 1978a,b). Fictive weight support can also be present in the contralateral limb (Perreault et al, 1999). Scratch can be elicited in cervical transected animals and in the absence of propriospinal feedback (Sherrington, 1910; Deliagina et al, 1975; 1981), demonstrating that this rhythmic activity is generated by spinal mechanisms.

Itch and the scratch reflex

Itch is defined as an unpleasant skin sensation that triggers the desire to scratch. In spite of their obvious relationship, and although cutaneous stimulation is used to activate the scratch CPG in the lumbar cord, how the "itch" signal actually triggers the scratch response has remained largely unexplored. However, considerable progress has recently been made regarding the peripheral and central mechanisms involved in the perception of itch and its distinction from pain (see Davidson & Giesler, 2010; Patel & Dong, 2010; Bautista et al, 2014; LaMotte et al, 2014; Akiyama & Carstens, 2014 for recent reviews). In the spinal cord, two seminal reports (Sun & Chen, 2007; Sun et al, 2009) identified the gastrin releasing peptide (GRP) and its receptor (GRPR) as the itch-specific mediators in rodents. GRP is expressed in a subset of sensory neurons with terminals in dorsal horn laminae I and II, where spinal neurons expressing GRPRs locate. These studies showed that mutant mice lacking GRPRs or mice with selective ablation of GRPR-positive neurons display dramatic reductions in scratch behaviour in response to diverse pruritogenic stimuli, but have normal tactile and pain responses. Further, intrathecal administration of the GRPR agonist, GRP_{18-27}, to the lumbar cord of wild-type mice induced scratching in a dose-dependent manner (Sun & Chen, 2007). Although subsequent studies have implicated substance P (Carstens et al, 2010), naturietic polypeptide B (Mishra & Hoon, 2013) and opioids acting at isoform 1D μ-opioid receptors (Liu et al, 2011) as itch-specific messengers, GRP appears to be a key mediator of itch transmission in the spinal cord. Whether GRP triggers scratching in cats is not known; however, a number of pharmacological strategies can elicit this reflex in the *in vivo* preparation.

Eliciting fictive scratch in cats

In decapitated and decerebrate cats, fictive scratch can be elicited by mechanical, thermal or electrical stimulation of the skin around the ear, head or neck (Sherrington, 1910). Feldberg and colleagues found that topical tubocurarine application to the dorsal surface of the first and second cervical (C1 and C2) segments of the spinal cord elicited spontaneous bouts of scratch or facilitated the scratch elicited by cutaneous stimulation (Feldberg & Fleischhauer, 1960; Domer & Feldberg, 1960). Electrical stimulation of these segments can also trigger the reflex (Sherrington, 1910); however, when this method fails, additional chemical stimulation is required for successful induction of scratching (Deliagina et al, 1975). Today, most studies evoke fictive scratch after placing small pieces of cotton soaked in 0.05 – 0.3% tubocurarine in C1-C2 segments and following manual stimulation (i.e. touching, rubbing, pinching) of the pinna. Strychnine (0.5%) or bicuculline (1-2 mg/ml) have also been topically used on cervical segments to facilitate manually-triggered scratching (Deliagina et al, 1981; Degtyarenko et al, 1998).

Do cat locomotion and scratch share common mechanisms?

Locomotion and scratch are different rhythmic motor behaviours that share features such as having a CPG structure within the spinal cord, and a pattern consisting of flexor and extensor alternation. Differences include: (1) variations in some muscle synergies, (2) scratch is a faster movement with shorter cycle periods, (3) postural bias is towards extension for locomotion and flexion for scratch, (4) initiation of scratch in experimental preparations does not require electrical stimulation of the brainstem or systemic drug administration and (5) locomotion includes the movement of all four limbs, whereas scratch involves rhythmic

activation of only one hindlimb, implicating simultaneous modulation of rhythmic and non-rhythmic activity on distinct sides of the spinal cord.

Interneuron and motoneuron recordings during transitions from locomotion to scratch in the same cat preparation show preservation of the firing pattern of these neurons within the cycle, suggesting that a common spinal mechanism, operating in different modalities, drives firing in both behaviours, and thus that locomotion and scratch share a common CPG structure (Deliagina et al, 1975; 1981; Berkinblit et al, 1978a,b; Gelfand et al, 1988). Differences in the activity pattern of some of these neurons, however, point to specialized control mechanisms for each movement (Degtyarenko et al, 1998; Berkowitz, 2008). Similarly, activity-dependent labeling of the interneurons activated during locomotion and scratch in cats show some degree of overlapping in areas near the central canal, although locomotor-related neurons are concentrated in more medial areas and scratch-related neurons in more lateral areas (Barajon et al, 1992; Dai et al, 2005). Evidence that locomotion and scratch are mediated by a circuitry consisting of both shared and specialized components has also come from studies assessing the modulation of sensory and supraspinal inputs on motoneurons during these behaviours (Perreault et al, 1999; Degtyarenko et al, 1998; Cote & Gossard, 2003; Frigon & Gossard, 2010), studies assessing the effect of afferent stimulation on motor rhythm (Perreault et al, 1999; Frigon & Gossard, 2010) as well as analyses of the characteristics of spontaneous deletions occurring during fictive locomotion and scratch (Lafreniere-Roula & McCrea, 2005). Intracellular recordings of cat lumbar motoneurons have shown that the excitability of these neurons is modulated in a similar fashion during fictive locomotion and scratch (Krawitz et al, 2001; Brownstone et al, 1992, 1994; Power et al, 2010). However, whether the neuromodulatory systems mediating these effects are also shared is not known. Compared to

the decerebrated cat preparation, *in vitro* preparations have some features that are advantageous for the pharmacological assessment of the neuromodulators involved in the control of motoneuron activity.

The *in vitro* neonatal rat spinal cord preparation

In spite the immaturity of rats at birth, the *in vitro* spinal cord preparation contains all anatomical and functional nervous structures related to motor control and thus it has been extremely useful for the study of the cellular mechanisms of mammalian locomotion. A rapid maturation of the rat motor system occurs during the first 2 postnatal weeks, including morphological and electrical changes in motoneurons and their inputs. The following section briefly summarizes information about the suitability of the *in vitro* neonatal rat spinal cord for the study of motor behaviour (for reviews see Vinay et al, 2000; Clarac et al, 2004).

The neuronal circuitry involved in the generation of locomotion, as well as the sensory and supraspinal systems that regulate its activity develop at late embryonic stages and their connectivity is refined during the first postnatal week. During postnatal development, motoneurons increase in size while their responsiveness to synaptic inputs decreases (Vinay et al, 2000). Increases in soma area, as well as decreases in membrane resistivity lead to reductions in motoneuron input resistance (R_{in}) with age. These changes correlate with a more than 5-fold increase in rheobase values (i.e. amount of current needed to initiate firing) in adult motoneurons. The increased excitability of these neurons at early postnatal stages is thought to contribute to the spontaneous activity observed in spinal networks at this age (Vinay et al, 2000).

Developmental changes in the ionic currents determining firing also contribute to motoneuron maturation. Action potentials appear at embryonic day 15 and postnatal changes in its properties mainly include amplitude increase, duration decrease, generation of the afterhyperpolarization (AHP) and V_{th} shift to more negative values (from -35.0 mV to -47.0 mV) without concomitant changes in resting membrane potential (resting V_m) (Gao & Ziskind-Conhaim, 1998). These adaptations are the result of increases in the density of sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) voltage-gated ion channels. Fast Na^+ current maturation increases the action potential rising rate and amplitude; moreover, a negative shift in the activation threshold of this current accounts for hyperpolarization of V_{th} after birth (Gao & Ziskind-Conhaim, 1998). Postnatal changes in K^+ conductances facilitate repetitive firing by increasing action potential repolarization rate, shortening spike duration and defining the AHP (Vinay et al, 2000). Ca^{2+} currents mediate a pronounced after-depolarization in the first few days after birth; however, after this stage Ca^{2+} currents affect repetitive firing instead of action potential shape (Vinay et al, 2000).

The *in vitro* neonatal rat spinal cord preparation offers several advantages for the study of motor behaviour including: (1) the spinal cord is quickly isolated and its small diameter permits enough 95% O_2/5% CO_2 diffusion to allow survival *in vitro* for several hours. (2) Bath applied pharmacological agents reach neurons directly due to the absence of blood-brain-barrier, and the incomplete myelination of the CNS. (3) Experimental control of the extracellular ionic composition facilitates the manipulation of the system excitability. (4) The preparation allows various arrangements to for example, selectively activate particular regions of the spinal cord through the use of acetate film and Vaseline barriers (i.e. partitioned bath approaches).

Neonatal rat locomotion

Initial locomotor experiments using the neonatal rat preparation performed by Smith & Feldman (1987) and Kudo & Yamada (1987) used spinal cords with the forelimbs and/or hindlimbs attached to characterize their movements. Locomotion consisted of ipsilateral (flexor/extensor) and contralateral (left/right) alternation of the limbs occurring at frequency of ~1 Hz. This frequency is consistent with real air-stepping of pups at this age (Clarac et al, 1998) and is ~2-4 times slower than adult rat locomotion (Gruner & Altman, 1980; Gruner et al, 1980). It should be noted, however, that the frequency of the locomotor-like activity produced *in vitro* varies largely with the characteristics of the chemical or electrical stimuli used to elicit it experimentally, ranging from 0.04 to 1 Hz and typically occurring at ~0.2 Hz (Clarac et al, 2004). By simultaneously recording muscular activity and ventral root (VR) activity in the same leg, Cazalets et al (1992) showed that the discharge of the second to third lumbar (L2-L3) VRs reflected mostly flexor muscle activity whereas the L5 VRs reflected mainly extensor muscle activity. Since then, hindlimb "locomotor-like" activity (i.e. recorded from VRs) has been characterized in the totally isolated spinal cord preparation as consistent with L2/L5 VRs bursts that alternate ipsi- and contra-laterally (cycle duration ~0.4-2.0s). However, when using these criteria for defining motor output, one must recognize that use of VR output has limitations in detecting and differentiating extensor and flexor motor output (Cowley & Schmidt, 1994).

Eliciting locomotor-like activity in neonatal rat preparations

Bath application of agonists for most neurotransmitter systems, including excitatory amino acids (glutamate, aspartate, NMDA and kainate), 5-HT, DA, NA and ACh, has the

ability to induce locomotor-like activity in the *in vitro* neonatal rat spinal cord (reviewed in Schmidt & Jordan, 2000; Clarac et al, 2004; Jordan et al, 2008, Miles & Sillar, 2011). When most of these transmitters are applied alone, however, the rhythmic activity that ensues does not last more than a few minutes and/or the activity pattern is not well coordinated. For this reason, most studies attempting to evoke sustained, well-coordinated locomotor-like activity utilize combinations of these compounds, typically 5-HT and NMDA. Also, although exposure of these two neurochemicals to any segment of the spinal cord can produce rhythmic activity (Cowley & Schmidt, 1997), most studies apply neuroactive compounds to lumbar segments of the spinal cord.

Electrical (Astuta et al, 1988; Zaporozhets et al, 2004) or chemical (Zaporozhets et al, 2006) stimulation of the brainstem also elicits stable locomotor-like activity in neonatal rats. Similarly, VLF stimulation triggers this behaviour (Magnuson et al, 1995). Electrical stimulation of lumbar or coccygeal dorsal roots (Whelan et al, 2000), or lumbar VRs in disinhibited cords (Mentis et al, 2005; Bonnot et al, 2009) has also been used to elicit locomotor patterns in *in vitro* neonatal mouse preparations.

Both adult decerebrate cat and neonatal rat preparations have been instrumental for the understanding of the mechanisms controlling rhythmic movements such as locomotion and scratch. Because these preparations do not require anaesthetics to be administered during the recording period, they have the capability to generate reproducible motor output and thereby have allowed the study of motoneuron firing behaviour prior to, during and after the generation of motor activity (e.g. as in Brownstone et al, 1992, 1994; Schmidt, 1994; Krawitz et al, 2001; Gilmore and Fedirchuk, 2004; Power et al, 2010). These investigations have

revealed that motoneuron excitability is dynamically adapted to match motor tasks, as reviewed next.

Dynamic regulation of motoneuron excitability during motor output

Persistent Inward currents

As described in previous sections, early studies using motoneuron recordings in cat preparations set a classical view of spinal motoneurons as passive integrators of synaptic input. After a series of experiments by Schwindt & Crill (1977, 1980a,b), a new picture emerged based on observations that the membrane potential of cat motoneurons had two stable states, one at rest and one at more depolarized values (e.g. resting V_m and plateau potential), a property known as bistability. In the absence of plateau potentials, motoneuron firing frequency is proportional to the amount of input injection. In contrast, the presence of plateaus allows amplification of excitatory inputs so that acceleration in firing frequency occurs during constant current injection. Schwindt & Crill (1977) demonstrated that plateaus and self-sustained firing were mediated by a negative slope conductance in the current-voltage relationship during a slow depolarizing voltage ramp. This conductance consisted of a voltage-dependent non-inactivating or slow-inactivating Ca^{2+} current, known as Ca^{2+}-persistent inward current (Ca^{2+}-PIC) (Schwindt & Crill, 1980a,b). These findings were quickly followed by reports from Hultborn and colleagues that, also in *in vivo* cats, motoneuron bistable behaviour could be elicited by brief activation of sensory afferents or depolarizing pulses, that it produced enhanced motoneuron excitability and thus long-lasting muscle contraction, and that this phenomenon was dependent upon descending

monoaminergic innervation (Hounsgaard et al, 1984, 1988; Crone et al, 1988; Conway et al, 1988).

Since these studies, PICs and their role in motoneuron excitability have been intensely investigated (reviewed in Heckman et al, 2003, 2009; Kim et al, 2014). PICs mainly originate from distal dendrites and in addition to their Ca^{2+} component (mediated by L-type ($Ca_V1.3$) Ca^{2+}-channels) other currents, such as a persistent Na^{2+}-current and a non-selective cation current, also contribute to their generation. Several lines of evidence suggest that plateau potentials play an important role in the amplification of synaptic input to motoneurons during motor behaviour. Consistent with PIC activation, voltage-dependent conductances emerge during fictive locomotion and scratch in decerebrated cats, enhancing motoneuron responses to depolarization (Brownstone et al, 1994; Power et al, 2010). In agreement with this, application of Ca_V1-channel blockers reduces the amplitude and duration of rhythmic activity in *in vitro* cords from juvenile mice (Jiang et al, 1999). Dendritic PICs thus are important determinants of motoneuron excitability during movement that can combine with changes in other motoneuron electrical properties.

Afterhyperpolarization

The hyperpolarization that follows the repolarization phase of action potentials, referred as afterhyperpolarization (AHP), is another parameter shown to be altered during motor activity. AHP duration inversely correlates with firing rate, and thus the impact of this property on motoneuron excitability is usually assessed by the slope of frequency-current plots (f/I slope). Ca^{2+}-activated K^+ channels mediate the AHP (Sah, 1996).

Using decerebrate cat preparations, Brownstone et al (1992) compared the repetitive firing produced by depolarizing current injection into motoneurons and the firing observed during fictive locomotion. They observed that, during similar interspike trajectories and at similar membrane potentials, the AHP amplitude during fictive locomotion was strongly reduced compared with AHPs from spikes evoked by current injection at rest. Further, they showed that although the f/I relationship was lost in most motoneurons during fictive locomotion, the firing rates during this behaviour were significantly larger than those achieved by maximal current injection (45 and 39 Hz, respectively). In one neuron, there was a dramatic increase in the slope of the f/I relation (from 0.8 to 2.9 impulses s^{-1} nA^{-1}), demonstrating that AHP amplitude reductions enhance repetitive firing during motor behaviour. Similarly, Schmidt (1994) reported a significant AHP amplitude decrease during chemically-induced locomotor-like activity in neonatal rat motoneurons. More recently, an analogous and reversible AHP reduction was reported to occur during fictive scratch in cat preparations (Power et al, 2010). Studies by Schmidt (1994) and Power et al (2010) suggested that the AHP suppression and decreased interspike intervals associated with rhythmic activity were not the result of a passive increase in membrane conductance, but rather that specific mechanisms mediated this effect. Evidence for such mechanism came from investigations in neonatal mouse motoneurons in which activation of type 2 muscarinic (M_2) receptors lead to reductions in the SK-type Ca^{2+}-activated K^+ conductance mediating the AHP, thus decreasing its amplitude and producing an increase in the f/I slope (Miles et al, 2007). AHP inhibition therefore provides an additional mechanism to facilitate motoneuron repetitive firing during movement.

Motoneuron recruitment

Given the importance of motoneuron activation for the production of movement, the factors that determine the recruitment of these neurons has been an area of sustained interest in motor research. As reviewed by Cope & Pinter (1995), motoneuron recruitment depends on a constellation of cellular (e.g. cell size, R_{in}, time constant) as well as synaptic (input distribution, organization and efficacy) factors. According to Ohm's law (current = voltage/resistance), the neuronal V_{th} and R_{in} determine the amount of current required to initiate firing. Consequently, the V_{th} constitutes one key cellular factor determining whether a motoneuron is activated or not, making this parameter a good target for neuronal excitability regulation.

The Voltage Threshold

Definition

A motoneuron is recruited into firing when its membrane potential is displaced from its value at rest to a particular value from which the action potential initiates, called the voltage threshold (V_{th}). Action potential generation requires a depolarizing stimulus of sufficient amplitude to overcome the leak current that restores the resting V_m. Also, the excitation must be fast enough to prevent accommodation due to Na^+ channel inactivation. In subthreshold depolarizations, the current carried by Na^+ entering the neuron is smaller than the K^+ current flowing out, so that the membrane potential returns to its resting level and firing does not occur. When the V_{th} is reached, the rate of K^+ outflow is slower than the rate of Na^+ inflow causing the sudden activation of voltage-gated Na^+ channels. A rapid Na^+ entry driven by concentration and voltage gradients produces further depolarization and activation

of adjacent Na^+ channels, triggering a positive feedback loop of depolarization that underlies the action potential (Hodgkin & Huxley, 1952; Coombs et al, 1955). A more specific definition of V_{th} thus is the value of membrane potential at which the inward Na^+ current overcomes the outward K^+ current and an action potential is initiated.

The axon initial segment

In their pioneering studies using intracellular recordings of cat motoneurons, Eccles and colleagues investigated the origin of action potential initiation within the neuronal membrane (Brock et al, 1953; Coombs et al, 1957a,b). By recording from intra-somatic and intra-axonic sites, and eliciting firing using different approaches (e.g. antidromic, monosynaptic or intracellular stimulation), these investigators postulated that depolarizing stimuli invaded 3 distinct areas of the motoneuron with different thresholds for spike generation: the axon initial segment (AIS) membrane, the nodal axonal membrane (so called medullated axon), and the somato-dendritic (SD) membrane (Coombs et al, 1957a). Based on the observations that: (1) the AIS V_{th} is the lowest (requiring a depolarization of 10 mV whereas the soma requires 30 mV), (2) the AIS spike occurs first during monosynaptic or direct intracellular excitation, (3) the AIS spike can occur in the absence of the SD spike and it can alone give rise to action potentials and (4) the AIS spike collides with antidromic action potentials, Coombs et al (1957b) concluded that the AIS, the structure at the juncture between the soma and the axon, is the site were action potentials are initiated.

The high density of Na_V channels in the AIS (Catterall, 1981; Kole et al, 2008) as well as their segregated distribution within this compartment enables the AIS to integrate all synaptic inputs and initiate firing. Using elegant immunohistochemical, electrophysiological

and computational modeling techniques, Hu et al (2009) demonstrated that due to their strategic location far from the soma in the distal AIS (30-50 µm from the soma) and their low threshold for activation, $Na_V1.6$ channels determine the low V_{th} for action potential initiation of rat pyramidal neurons. $Na_V1.2$ channels, on the other hand, are responsible for spike backpropagation and generation of SD potentials due to their high threshold for activation and their location close to the soma in the proximal AIS (10-20 µm from the soma). A similar complementary gradient of $Na_V1.6$ and $Na_V1.1$ channel subtypes in distal and proximal domains of the AIS, respectively, is present in mouse spinal motoneurons (Duflocq et al, 2008). As Hu et al (2009), other studies have provided mechanistic explanations of Coombs et al (1957a,b) hypothesis that the V_{th} at the soma is ~15 to 20 mV more depolarized than at the AIS, making this structure the site where action potentials are initiated (see reviews by Dulla & Huguenard, 2009; Kress & Mennerick, 2009).

Because action potentials are initiated at the AIS, the V_{th} values measured at the soma (the presumed recording site) may represent overestimates of the actual axonal V_{th}. However, because soma diameter and AIS are positively correlated (Cullheim, 1978), the proportion of the overestimation is probably similar across differently sized motoneurons. Therefore, in investigations on the modulation of V_{th}, reproducible changes in this parameter as index of neuron excitability, where each individual cell is compared to itself during different conditions, are more important than the absolute values of the recorded V_{th} as compared across different cells.

Voltage threshold hyperpolarization

Negative shifts in the membrane potential at which action potentials start are referred as "V_{th} hyperpolarization" or "V_{th} lowering". Once V_{th} hyperpolarization occurs, the amount of depolarization required to elicit firing is reduced, so recruitment is facilitated and neuronal excitability is enhanced. V_{th} hyperpolarization can be seen in response to long-term sensitization training of the withdrawal reflex in the mollusc *Aplysia* (Cleary et al, 1998) and endurance treadmill training in adult rats (Beaumont & Gardiner, 2003). A similar adaptation occurs following acute increases in motor activity.

The rapid modulation of V_{th} as means to adjust motoneuron excitability to a behavioural state was first demonstrated by Krawitz et al (2001). By using every neuron as its own control and comparing their V_{th} values prior to and during MLR-induced locomotion in decerebrate cats, these researchers showed that motoneuron V_{th} hyperpolarization occurs during fictive locomotion. This change was robust (mean -8.0 mV) and occurred in all neurons examined, including motoneurons innervating flexor and extensor as well as slow and fast contraction muscles. Further, V_{th} hyperpolarization appeared within seconds after MLR-evoked locomotion and it could occur prior to motoneuron recruitment. This change was reversible, recovering quickly after a bout of locomotion. V_{th} hyperpolarization occurred without concomitant changes in action potential amplitude or duration, and because this effect was evident for the very first action potential elicited, it was not a consequence of repetitive firing. Moreover, V_{th} lowering did not vary with the rhythmic fluctuations of membrane potential observed during locomotion and thus it was not the result of these oscillations. A computational study conducted by Dai et al (2000), strengthened the idea that membrane potential trajectory alone does not account for the locomotor-induced V_{th} hyperpolarization,

implicating instead the involvement of neuromodulatory mechanisms on Na^+ and K^+ conductances at the AIS. Very recently, MacDonell et al (2015) showed that MLR-induced locomotion in *in vivo* adult rats induced extensor motoneuron V_{th} hyperpolarization in a similar extent to that observed in cats. Interestingly, this change occurred in the 2-10 s-long tonic period immediately preceding the start of locomotion and prior to motoneuron involvement in motor output, suggesting that motor pools are primed by neuromodulators for movement production.

Neonatal rat motoneurons display V_{th} changes during brainstem-induced locomotor-like activity analogous to those reported in adult cats and rats (Gilmore & Fedirchuk, 2004). In the *in vitro* brainstem-spinal cord preparation, V_{th} lowering (mean -6.0 mV) occurs during electrical stimulation of the ventromedial medulla independently of whether locomotor-like activity is produced or whether other VR activity patterns are generated, and even in the absence of motor output. Further, graded brainstem stimulation showed that intensities subthreshold for inducing rhythmic activity still elicit motoneuron V_{th} hyperpolarization, again suggesting neuromodulator-induced regulation of motoneuron excitability prior to the start of motor behaviour.

State-dependent modulation of motoneuron V_{th} is not exclusive of locomotion. In decerebrate cats, V_{th} hyperpolarization also occurs during fictive scratch (Power et al, 2010). Similar to the changes reported during fictive locomotion, V_{th} hyperpolarization during scratch was strong (mean -5.8 mV), occurred in most motoneurons studied (80%) and significantly reduced the amount of current required to allow recruitment. Also, this effect persisted throughout both the depolarizing and hyperpolarizing phases of scratch-induced membrane fluctuations and was not associated with changes in action potential height or

duration. The change in V_{th} during scratch was reversible and occurred prior to the start of tonic (approach phase) or rhythmic network activity. Interestingly, restriction of V_{th} lowering to motoneurons innervating the scratching limb suggested that modulation of motoneuron excitability can be site and state-specific.

Together, these studies indicate that V_{th} hyperpolarization constitutes a mechanism used by the motor system to facilitate motoneuron firing prior to and during distinct motor behaviours. V_{th} depolarization, on the other hand, also provides adaptive advantages during both chronic and acute states (see next section).

Voltage threshold depolarization

An additional remarkable observation of the cat fictive scratch experiments reported by Power et al (2010) was that: whereas the V_{th} of extensor motoneurons hyperpolarizes during ipsilateral scratch, these same neurons could show the opposite effect (e.g. V_{th} depolarization) during fictive weight support that occurs during contralateral scratch. Similar to the state-dependent V_{th} hyperpolarization, V_{th} depolarization occurred before the onset of firing elicited by scratch or weight support, and recovered a few seconds after the rhythmic bouts had ended. In contrast, V_{th} depolarization was associated with reversible reductions in action potential amplitude. Further, comparison of the firing frequencies during fictive weight support and the excitatory phase of scratch drive potentials showed that lower firing rates were attained during the former, overall suggesting decreased levels of motoneuron excitability during weight support. Perreault (2002) also reported a differential effect of these two behaviours on motoneuron properties. Whereas significant reductions in the R_{in} and membrane time constant of extensor motoneurons occur during all phases of scratching, these

parameters do not change during fictive weight support, even at similar membrane depolarization levels. This difference was attributed to variations in the synaptic drive between the two patterns, and particularly to a stronger inhibitory drive during fictive scratch. Together, these studies suggest that modulation of motoneuron firing is behaviour-specific and that these mechanisms may operate simultaneously in situations in which both tonic and rhythmic motor patterns coexist.

V_{th} depolarization is not a newly described phenomenon. A pronounced rise of cat motoneuron V_{th} occurs during sustained firing evoked by synaptic input (Kolmodin & Skoglund (1958) or intracellular current injection (Granit et al, 1963), to the extent that tonic firing is absent at moderately high levels of membrane depolarization. This phenomenon, known as accommodation, highlights the dependence of V_{th} on the depolarization rising rate. During slow depolarizations, time allows activated Na^+ channels to undergo inactivation and K^+ channels to restore the resting V_m, resulting in a delay of action potential initiation (Schlue et al, 1974; Schwindt & Crill, 1982).

Chronic depolarization of motoneuron V_{th} also constitutes a plastic adaptation to decreased levels of motor activity. Operant conditioning to decrease the size of the H-reflex in monkeys (Carp & Wolpaw, 1994) and hindlimb unweighting for 2 weeks in adult rats (Cormery et al, 2005) both induce V_{th} depolarization (mean +3.0 mV and +5.0 mV, respectively) of motoneurons from trained compared with non-trained limbs. Further, motoneuron V_{th} depolarization (mean +1.7 mV) and increased rheobase values occur following 6 weeks of spinalization of adult cats (Hochman & McCrea, 1994). Similarly, 4 weeks after spinalization hindlimb motoneurons display V_{th} depolarization (mean +4.0 mV) in adult rats (Beaumont et al, 2004). Interestingly, this last study showed passive cycling

exercise (1 hour/day, 5 days/week, for 4-5 weeks) prevented the V_{th} depolarization induced by spinalization, suggesting that decreased and increased activity levels exert opposite effects on motoneuron excitability adaptations.

The mechanisms underlying V_{th} adaptations in both the depolarizing and hyperpolarizing directions are not well understood. However, pharmacological and modeling studies have shed light onto the putative conductances subjected to modulation and underlying these changes.

Mechanisms for voltage threshold plasticity

Dai et al (2002) developed a five-compartment neuron model based on the physiological properties of cat spinal motoneurons to determine the ionic bases of the locomotion-induced V_{th} hyperpolarization described in cats. This model included most voltage-dependent ion channels underlying the action potential waveform, including: transient (Na_T) and persistent (Na_P) Na^+ channels, Ca^{2+}-dependent K^+ channels (KCa_{BK} and KCa_{SK}), delayed rectifier K^+ channels (K_{DR}), inward rectifying channels (K_{ir} and I_h), leak K^+ channels (K_{leak}), transient outward channels (K_A) and L-, N- and T-type Ca^{2+}-channels (Ca_L, Ca_N, Ca_T). Selective modulation of Na^+ conductances, and in lesser degree K^+ conductances, played a dominant role in acute V_{th} hyperpolarization associated with the locomotor state. Specifically, V_{th} hyperpolarization could be reproduced by: (1) 50% increase of AIS maximum Na_T conductance, (2) 3.0 mV hyperpolarizing shift in the voltage dependency of AIS Na_T conductance, (3) 70% reduction in AIS K_{DR} conductance and (4) 5 mV depolarizing shift in the voltage dependency in AIS K_{DR} conductance. Modulation of these conductances, by yet unknown neuromodulatory pathways, mimicked the *in vivo* motoneuron V_{th} hyperpolarization

in the extent of that occurring during fictive locomotion while leaving action potential height and duration unchanged. Cormery et al (2005) used this model to assess the putative ionic mechanisms underlying motoneuron V_{th} depolarization as well as changes in other electrical properties induced by limb unweighting. Analogous to the state-dependent V_{th} hyperpolarization, reductions in Na^+ conductances at the AIS and soma (25 and 15 %, respectively) or increases in K_{DR} conductance (55 and 42%, respectively) in these areas accounted for the motoneuron property changes induced by limb suspension. Gardiner et al (2006) reached similar conclusions from modeling chronic changes induced by endurance training in rat motoneurons.

Power et al (2012) recently confirmed that a facilitation of the Na^+ inward current at subthreshold membrane potentials can mediate motoneuron V_{th} hyperpolarization. Using veratridine, a plant alkaloid neurotoxin that shifts the activation voltage of Na^+ channels towards negative values, they demonstrated that direct modulation of Na^+ channels consistently induces V_{th} lowering in neonatal rat lumbar motoneurons. An indirect effect in V_{th} via enhancement of PICs was ruled out by observations that veratridine effects on PICs had a direction and timing different from the effects on V_{th}. The extent of veratridine-induced V_{th} hyperpolarization mimicked that reported during locomotion and scratch, strengthening the idea that Na^+ channels can be targets for neuromodulatory action during motor output. The control of neuronal excitability through modulation of Na^+ channels has been demonstrated in a variety of cells (Catterall, 2000; Cantrell & Catterall, 2001; Chen-Izu et al, 2015).

As reviewed by Dai et al (2009b), studies in different neurons have implicated the protein-kinase C (PKC) pathway in modulating Na^+ currents by reducing their peak amplitude without affecting their voltage dependence. However, it was shown in neocortical neurons that

PKC activation led to negative shifts in Na_T current activation and inactivation curves (Franceschetti et al, 2000) consistent with the V_{th} hyperpolarization induced by this pathway (Astman et al 1998). Dai et al (2009b) explored the effects of PKC modulation of Na^+ currents and V_{th} in spinal neurons from neonatal rat spinal cord slices. In these neurons, PKC activation induced an increase in Na_T half width and reductions in Na_T amplitude and maximal rise and decay rates with variable effects on V_{th}. In most cells (57%) V_{th} remained unaltered whereas 20% and 9% of cells showed V_{th} depolarization and hyperpolarization, respectively. They concluded that PKC activation reduces the excitability of spinal ventral neurons via mixed effects including, but not restricted to, a reduction in Na_T amplitude, and to a lesser extent, a depolarization of Na_T and V_{th}.

While AIS Na_V channels seem to be subject to short-term plasticity, recent studies in supraspinal neurons have revealed novel mechanisms for long-term activity-dependent V_{th} adaptations through changes in the AIS structure. V_{th} depolarization in response to chronic increased neuronal activity *in vitro* is produced by distal relocation of the AIS (Grubb & Burrone, 2010), whereas deprivation of sensory input *in vivo* produces compensatory V_{th} hyperpolarization via a >50% increase in AIS length (Kuba et al, 2010). In addition to changes in AIS position and size, Muir & Kittler (2014) showed that the diffusion dynamics (lateral mobility and synaptic clustering) of receptors present in the AIS can also be subject to chronic plasticity. It is thus becoming evident that the AIS constitutes a plastic hotspot where neuromodulatory influences can directly regulate neuronal excitability (see reviews by Grubb et al, 2011; Adachi et al, 2014). How modulators exert this control at the cellular and network levels is the subject of the following section.

Role of neuromodulators in the control of neuron and network activity

Modification of the ionic conductances underlying a neuron's firing behaviour as the examples described above is only one of the mechanisms used by neuromodulators to regulate motor output. Neural networks such as CPGs produce patterns of activity as a consequence of both the intrinsic properties of neurons in the circuit and their synaptic connectivity. Neuromodulatory inputs to CPGs can alter: (1) the activity of each neuron, thus defining neuronal composition of the active circuit, (2) the intrinsic properties of each neuron, thus defining their responsiveness to other inputs, and (3) the synaptic properties of the system by strengthening, weakening, activating, inactivating, reversing (or non-affecting) synapses, thus defining the functional connectivity of the circuit (Harris-Warrick, 1988). In the long term, neuromodulators also regulate the electrophysiological identity of neurons by controlling the expression of different ion channels (see Harris-Warrick, 2011 for review).

Attempts at identifying all the molecular targets of a single modulator in all neurons and synapses of a network have only been possible in invertebrate model systems given their relative simplicity. For example, the crustacean somatogastric ganglion (STG) that drives foregut rhythmic movements underlying the filtering of chewed food, contains only 14-31 neurons that have been systematically studied in isolation as well with each of their synaptic connections. Further, most of the neuromodulatory influences to the STG including descending projections, afferent inputs and hormones have been identified (Marder & Bucher, 2001). Studies using this system have provided key insights of neuromodulatory action on motor CPGs that are relevant to all CNS systems. One of the lessons learnt from this system as well as from vertebrates is that interpretation of the functional significance of the effects of

a modulator must consider how this compound is in turn regulated by other neuromodulators and the physiological state of the network it affects.

Metamodulation

Just as a neuromodulator can alter the effectiveness of neurotransmission, it can also alter the effectiveness of neuromodulation, a concept known as metamodulation or second order modulation. This control is exerted by promoting or preventing neuromodulator availability, and/or enhancing or inhibiting a modulator's effect at its targets. Very little is known about metamodulation. As reviewed in Dickinson (2006), direct application of some neurochemicals or activation of sensory inputs regulates modulatory neuron activity in the crustacean STG system. Also, heart muscle has been shown to provide metamodulatory input to the CPG located in the cardiac ganglion in this species via the retrograde messenger nitric oxide (NO). In *Xenopus* tadpoles, NO and NA facilitate glycinergic inhibition to slow the swimming frequency; however, NO exerts this action indirectly via NA pathways (increasing endogenous spinal NA release), placing NO above NA in the metamodulatory hierarchy (McLean & Sillar, 2004). Similarly, the lamprey locomotor CPG is strongly modulated by tachykinins such as SP which in turn are metamodulated by co-released 5-HT and DA. In this system, 5-HT directly inhibits SP-mediated modulation, including short-term presynaptic facilitation of glutamate release and long-term postsynaptic potentiation of glutamatergic responses, whereas DA adds on 5-HT postsynaptic effects but it blocks the 5-HT presynaptic component. The interaction between DA and 5-HT thus provides a gating mechanism for short-term SP-induced plasticity and constitutes an example of "modulation of metamodulation" (Svensson et al, 2001).

State-dependent effects of neuromodulators

It is becoming increasingly evident that networks as well as their component neurons respond to the same modulator differently depending on the state of the network activity. The term "network state" is usually used to refer to the activity of multiple interconnected neurons at a moment, determined by the dynamics of the cellular properties of the neurons intrinsic to the network and their synaptic connections (Fontanini & Katz, 2008). Changes in these properties, occurring within short or long time scales, profoundly impact the response of neurons to their ionotropic or metabotropic inputs (Nadim et al, 2008). The state of a network is in turn modified by changes in the balance of synaptic inputs to the network, the effects of neuromodulators, the history of network activity, or high-order factors such as behavioural states including sleep/wakefulness, attention, learning, hunger or social status (reviewed in Nadim et al 2008). The dynamic nature of the network state as well as difficulties in the manipulation or even identification of network states has complicated the assessment of how this factor shapes neuronal processing. However, a growing number of physiological and computational studies highlight that individual neuron and network responses to perturbations are a function of network states.

In the STG system, several modulators show state-dependent effects so that the activity of the network determines the extent of modulator action. For example, the neuropeptide proctolin dramatically increases the rhythmic frequency of the crustacean STG in preparations initially inactive or slowly active (<1 Hz) but it has virtually no effects in preparations with a high starting frequency (>1 Hz) (Nusbaum & Marder, 1989). Other peptides have similar excitatory or inhibitory effects on pyloric STG rhythm depending on the initial frequency (Skiebe & Schneider, 1994; Weimann et al, 1997; Ma et al, 2009; Szabo et

al, 2011). Furthermore, 5-HT in this system can produce increases, decreases or no change of cycle frequency in preparations without different starting frequencies (Spitzer et al, 2008). These variable effects were instead related to differences in the balance among 3 separate components of the response to 5-HT mediated by distinct transduction cascades (inositol phosphate, cyclic adenosine monophosphate, and an unknown pathway), so that preparation-to-preparation variability in each component determined the direction and magnitude of the 5-HT effect. Differences in baseline variability of neuronal networks (including both activity and inter-individual parameters) also explain discrepancies of neuromodulator effects in other systems (see Marder & Goaillard, 2006; Marder, 2011; Hamood & Marder, 2015 for review); however, reliable responses can also be obtained from networks with different parameters (Grashow et al, 2009).

In addition to network state, it should be kept in mind that neurons and networks receive simultaneous modulatory inputs from many different sources. Consequently, the relative contribution of a modulator can be critical under some conditions but trivial at other times (Harris-Warrick & Marder, 1991). Shifts in the reliance from one modulatory system to another have been demonstrated in the mammalian respiratory system (Doi & Ramirez, 2010) and it is possible that this phenomenon occurs in the spinal locomotor system when descending modulatory influences are removed or impaired.

Descending *versus* intraspinal modulation of motoneuron excitability

Neuromodulator systems controlling mammalian spinal motoneuron excitability can be classified according to the location of their projecting neurons into 3 categories: descending systems (including 5-HT, NA and DA), intraspinal systems (ACh) and systems originating

from both extra- and intra-spinal locations (including amino acids acting at metabotropic receptors, peptides, purines and NO).

The descending monoaminergic system

Serotoninergic inputs to the spinal cord originate from the brainstem in the raphe nuclei region (Dahlstroem & Fuxe, 1964; VanderHorst & Ulfhake, 2006) and from an area of the mid-medulla called the parapyramidal region (Liu & Jordan, 2005). After spinal transection, 2-10% of 5-HT (accounting for only 3-9 neurons) remains below the lesion (Carlsson et al, 1963; 1964) that is thought to be related with intraspinal modulation of sympathetic function (Newton et al, 1986; Newton & Hamill, 1988). Neurons in raphe nuclei mainly project to lumbar motoneurons via the ventral funiculi (Schmidt & Jordan, 2000). Approximately 1,500 5-HT boutons contact the somatodendritic membrane of individual motoneurons, accounting for 1-3% of the total synaptic inputs to these neurons (Alvarez et al, 1998). However, it has been shown that 5-HT acting at extra-synaptic receptors in the AIS (e.g. via spillover mechanisms) importantly contributes to the effects of this modulator in spinal motoneurons (Cotel et al, 2013), indicating that modulators do not necessarily require the presence of specialized synaptic structures to exert their effects.

As reviewed by Renkling et al (2000), Schimdt & Jordan (2000), and Miles & Sillar (2011), 5-HT enhances motoneuron excitability by: 1) depolarizing the resting V_m (by facilitating I_h and Ca_L channels, and inhibiting K_{ir} channels), 2) reducing the AHP (by inhibiting by KCa channels), 3) promoting plateau potentials (by facilitating Ca^{2+}- and Na^{+}-PICs and reducing K^{+} conductances), 4) facilitating NMDA-dependent oscillations (by reducing the voltage sensitivity of NMDA channels) and 5) hyperpolarizing the V_{th} (via

unknown mechanisms). Inhibitory effects of 5-HT, such as hyperpolarization of the V_m (Holohean et al, 1990; Wang & Dun, 1990; Zhang et al, 1991) or V_{th} depolarization (Cotel et al, 2013) in different motoneurons or within the same motoneuron (Perrier & Cotel, 2008) also exist, and are presumably mediated by different receptor subtypes from those mediating excitation (Perrier & Cotel, 2008; Schmidt & Jordan, 2000).

Noradrenergic and dopaminergic inputs to the spinal cord also originate from supraspinal centers, the locus coeruleus and the substantia nigra, for NA and DA respectively. These two amines share several modulatory mechanisms with 5-HT to regulate motoneuron behaviour and thus their dominant effect is also excitatory (Renkling et al, 2000; Miles & Sillar, 2011).

Challenged dependence upon descending systems

Hultborn and colleagues findings that bistable behaviour was eliminated following acute spinal transection and recovered via 5-hydroxytryptophan or L-DOPA administration in decerebrate cats first suggested the dependence upon descending monoaminergic inputs for regulation of motoneuron excitability (Hounsgaard et al, 1988; Crone et al, 1988). Later, Lee & Heckman (2000) showed that different levels of monoaminergic drive to motoneurons modified the amount of amplification of the synaptic input likely caused by activation of dendritic PICs. They used cat preparations with low (pentobarbithal-anesthetized), moderate (decerebrate) and high (decerebrate + NA agonist) monoaminergic input to demonstrate that, whereas in the enhanced modulatory state synaptic amplification is the highest, in the moderate and low states it is reduced by ~4- and 6-fold, respectively. Also, similar to Hounsgaard et al (1988), spinal transection abolished voltage-dependent amplification in this

study. In neonatal rodent motoneurons, monoamines induce V_{th} hyperpolarization (Fedirchuk & Dai, 2004; Han et al, 2007; Tartas et al, 2010). Gilmore & Fedirchuk (2004) showed in this preparation that cooling of the cervical cord or application of the 5-HT_2 antagonist ketanserin reversibly blocked the V_{th} hyperpolarization observed during brainstem-induced locomotion. These studies strengthened the idea that movement-dependent modulation of motoneuron excitability requires the activation of descending serotoninergic inputs.

Recently however, Power et al (2010) demonstrated that enhancement of motoneuron excitability associated with motor output persists following acute cervical transection in decerebrated cats. As described in previous sections, reversible voltage-dependent synaptic amplification, V_{th} hyperpolarization and AHP reduction in motoneurons occur during fictive locomotion and scratch. Power et al (2010) showed that all these state-dependent changes persist during fictive scratch in cats following complete spinal transection at the first cervical segment. Similar to spinal-intact preparations, changes in motoneuron properties were robust, reversible and consistent. This study revealed a previously unrecognized intraspinal system capable of regulating motoneuron excitability during motor behaviour independent of the activation of descending projections. The implications of this finding are extremely relevant for rehabilitation strategies after spinal cord injury: given that adequate production of movement likely depends on state-dependent adjustment of motoneuron excitability, restoration strategies can be developed to target neuromodulatory systems below the injury. The neuromodulatory identity of the propriospinal system regulating motoneuron excitability has not been determined and this provided the rationale for projects 1 and 2 in this thesis. The final sections of this introduction review the role of neuromodulatory systems intrinsic to the spinal cord in the regulation of motoneuron excitability.

Intraspinal systems

Glutamate and GABA are best known by their fast neurotransmitter action in the CNS and thus they have a widespread presence in the spinal cord that includes intraspinal, descending and sensory sources. These transmitters can act as neuromodulators by binding to G-protein coupled metabotropic receptors (group I-III metabotropic glutamatergic receptors and B-type GABA receptors, respectively). These receptors appear to be located primarily at presynaptic sites, where they regulate neurotransmitter release; very little is known about their postsynaptic actions in mammalian motoneurons (Miles & Sillar, 2011). For example, although activation of group I metabotropic glutamatergic receptors in spinal motoneurons induces V_{th} hyperpolarization, resting V_m depolarization and rheobase decrease, their net effect on motoneuron excitability is inhibitory due to induction of Na_T current reductions affecting action potential parameters and repetitive firing (Iwagaki & Miles, 2011). Peptides (e.g., SP, hormones), purines (adenosine triphosphate, adenosine), endocannabinoids (e.g., anandamide) and gaseous modulators (NO) have shown to play a role in spinal motor control, however, their actions on motoneuron firing behaviour, if any, remain unclear (Renkling et al, 2000; El Manira et al, 2008; Miles & Sillar, 2011). The spinal cholinergic system has been strongly involved in locomotor function and probably represents the strongest candidate for intraspinal modulation of motoneuron excitability.

Involvement of acetylcholine in spinal motor systems

The spinal cholinergic system and locomotor network function

In mammals, all ACh inputs to spinal neurons originate exclusively from within the spinal cord (McLaughlin, 1972; Sherriff et al, 1991; Sherriff & Henderson, 1994;

VanderHorst & Ulfhake, 2006). Intraspinal ACh sources include: motoneurons, preganglionic autonomic neurons, small dorsal horn cells scattered in laminae II-V, partition cells (lamina VII, between dorsal and ventral horns), and central canal neurons (lamina X) (Barber et al, 1984; Phelps et al, 1984; Borges & Iversen, 1986). Concomitantly, considerable expression of muscarinic receptors (and a 2-3 times lower density of nicotinic receptors) exists along the spinal cord, with highest densities in the ventral horn and around the central canal (Oguz Kayaalp & Neff, 1980; Gillberg et al, 1988; Wada et al, 1989; Yung & Lo, 1997; Khan et al, 2003).

Extensive evidence involves the spinal cholinergic system in mammalian locomotion. At early embryonic stages in rodents, motoneuronal ACh release contributes to the appropriate assembly of the spinal CPG circuitry (Hanson & Landmesser, 2003; Myers et al, 2005). Later at neonatal stages, ACh (and/or cholinesterase inhibitors) has the ability to induce locomotor-like activity, although in some preparations this activity consists of left/right alternation with synchronous flexor/extensor output (Cowley & Schmidt, 1994; see also Jordan et al, 2014). In adult cats and rats, studies combining activity-dependent markers, immunohistochemical staining and electrophysiological recording of individual neurons have reported activation of cholinergic interneurons in laminae VII and X during locomotion (Carr et al, 1994, 1995; Huang et al, 2000; Tillakaratne et al, 2014). Cholinergic neurons represent ~30% of the total locomotor-activated neurons (Carr et al, 1995) and this number increases by increments in load during locomotion via elevation of treadmill incline (Tillakaratne et al, 2014). Complementing these observations, studies in neonatal mice showed that ACh modulates the electrical properties of locomotor-activated neurons in laminae VII, VIII and X by altering V_{th}, AHP, resting V_m, R_{in}, action potential parameters and I_h (Dai et al, 2009a; Dai & Jordan,

2010). Interestingly, effects of ACh in some of these properties were opposite or additive to those of 5-HT, suggesting that these modulators act through separate mechanisms (Dai et al, 2009a). In addition to modulating firing behaviour, ACh directly activates commissural cells implicated in the control of locomotion (Carlin et al, 2006). Also, muscarinic receptor activation in sacrocaudal neurons with ascending lumbar projections has been shown to modulate locomotor amplitude and frequency in neonatal rats (Finkel et al, 2014; Etlin et al, 2014). Furthermore, anatomical and functional studies suggest that cholinergic inputs to motoneurons have important implications for their firing behaviour during movement.

Anatomy of cholinergic inputs to motoneurons

Cholinergic inputs to motoneurons were first described by their ultrastructure in early electron microscopy studies (Conradi, 1969; Conradi & Skoglund, 1969). These studies reported distinctively large (3-6 µm in diameter) synaptic terminals on the soma and dendrites of motoneurons, named C-boutons, that were characteristically associated with postsynaptic specialized structures of endoplasmic reticulum called subsurface cisterns. The cholinergic nature of C-boutons was confirmed more than a decade later by immunohistochemical studies using antibodies against choline-acetyl-transferase (ChAT), acetylcholinesterase (AChE) and vesicular acetylcholine transporter (VAChT) (Connaughton et al, 1986; Nagy et al, 1993; Li et al, 1995; Arvidsson et al, 1997). C-boutons account for less than 10% of the total of motoneuron synapses; however, due to their large size they contribute to ~20% of the total somatic synaptic input (Kellerth et al, 1979; Fyffe, 2001). Although initially described in cats (Conradi, 1969), C-boutons are present in brainstem and spinal motoneurons from all mammals examined to date (Bodian, 1975; Bernstein & Bernstein, 1976; Hamos & King,

1980; Wilson et al, 2004). Further, C-boutons appear to occur exclusively on alpha motoneurons, and not gamma motoneurons or other neuron types (Arvidsson et al, 1987; Welton et al, 1999), with higher densities in motoneurons innervating fast compared with slow muscles (Hellstrom et al, 2003). In rodents, C-boutons and most of their associated proteins are present at birth and they fully mature within the first 2 postnatal weeks (Phelps et al, 1984; Wetts & Vaughn, 2001; Wilson et al, 2004).

Several proteins, including receptors and ion channels have been shown to cluster at C-bouton synapses. The most remarkable is the presence of muscarinic M_2 ACh receptors (Hellstron et al, 2003; Muennich & Fyffe, 2004), but not other muscarinic receptor subtypes (Wilson et al, 2004) in the postsynaptic membrane, suggesting that M_2 receptors mediate ACh actions at this synapse. $K_V2.1$ potassium channels co-localize with M_2 receptor clusters (Muennich & Fyffe, 2004; Wilson et al, 2004). Also, postsynaptic KCa_{SK} channels of the SK2 subtype are present in all motoneurons whereas SK3 subtype expression restricts to small (presumably slow) motoneurons in rodents, suggesting that some specificity in C-bouton signaling exists according to motoneuron type (Deardorff et al, 2013). Presynaptically, C-boutons express $Ca_V2.2$ calcium channels (Wilson et al, 2004), ATP (P2X7) receptors (Deng & Fyffe, 2004) and nicotinic ACh receptors containing subunits alpha3, alpha4, alpha5 and beta2 (Khan et al, 2003) that together may regulate ACh release at these terminals.

Recent studies by Miles and collaborators (2007) showed that the neuronal source of C-boutons is restricted to a subpopulation of medial partition neurons expressing the transcription factor Dbx1, a marker of the developmentally defined V0 interneuron group, and located lateral to the central canal. In a comprehensive study, this group of researchers characterized the neurons originating C-boutons genetically, phenotypically, anatomically,

physiologically and behaviourally (Zaigoraiou et al, 2009). First, using genetic screening they found that another transcription factor, Pitx2, labels a similar subpopulation of V0 interneurons clustered longitudinally near the central canal. By generating mice in which Pitx2-positive neurons expressed a fluorescent protein, they showed that virtually all C-boutons originate from a subset of Pitx2-positive neurons expressing cholinergic markers ($V0_C$ neurons). Trans-synaptic labeling via injection of pseudo-rabies virus into hindlimb muscles revealed that the majority of projections from $V0_C$ neurons are to ipsilateral motoneurons, while ~30% of neurons project contra-laterally. A single $V0_C$ neuron formed ~1,000 synaptic contacts with individual motoneurons and innervated ~10 motoneurons. Functional assessment of Pitx2-positive neurons suggested that they may be targets of the same inputs received by motoneurons including descending, sensory and local signals (reviewed in Witts et al, 2014).

C-boutons are not the sole source of cholinergic inputs to motoneurons. A subset of S-boutons, small (<4 μm in diameter) synapses containing spherical vesicles, release ACh and establish connections with motoneurons (reviewed in Fyffe, 2001). Some of these terminals originate from homonymous motoneurons as recurrent collaterals, and are associated with fast excitatory transmission mediated by postsynaptic nicotinic receptor subtypes (Cullheim et al, 1977; Lagerback et al, 1981; Ichinose & Miyata, 1998). S-boutons in motoneurons are present from embryonic day 11 (E11) in rats and increase in numbers in the first postnatal weeks (Wetts & Vaughn, 2001). In contrast to C-boutons, S-boutons tend to contact distal dendrites, although both of these synaptic structures can also be present in the motoneuronal AIS (Kellerth et al, 1979).

In contrast with advances in the anatomy of cholinergic synapses, and spite that these studies point that ACh constitutes a major input to motoneurons, very little is known about their functional significance.

Cholinergic modulation of motoneuron excitability

Most studies assessing the effect of ACh on motoneuron electrical properties have supported a net excitatory postsynaptic role; however, the underlying specific mechanism(s) have remained obscure. Initial lumbar motoneuron recordings in cats showed that iontophoretic application of ACh caused a dose-dependent V_m depolarization (>5.0 mV) and changes in action potential parameters such as reduced amplitude and slower rise and decay times (Zieglgansberger & Reiter, 1974). The general muscarinic receptor antagonist, atropine, blocked the ACh-induced V_m depolarization in most neurons (Zieglgansberger & Bayerl, 1976). Subsequent studies in neonatal rats also reported depolarization of motoneurons induced by ACh involving muscarinic mechanisms (Evans, 1978; Jiang & Dun, 1986; Kurihara et al, 1993; Newberry & Connolly, 1989). However, other studies using neonatal, juvenile and adult rats implicated nicotinic instead of muscarinic receptors in ACh-induced depolarizations in both spinal and brainstem motoneurons (Blake et al, 1987; Zaninetti et al, 1999; Wang et al, 1991; Robinson et al, 2002; Ogier et al, 2004, 2008; Liu et al, 2005).

Studies assessing the presynaptic effects of ACh have also generated confusing results. In rat hypoglossal motoneurons, excitatory glutamatergic inputs are suppressed by muscarinic agents (Bellingham & Berger, 1996; Liu et al, 2005). In contrast, muscarinic (Bertrand & Cazalets, 2011) or nicotinic (Mine et al, 2015) agonists facilitate glutamatergic excitatory postsynaptic currents in rat lumbar motoneurons. Further, α-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid (AMPA) glutamatergic inputs to mice lumbar motoneurons originating from supra-spinal and inter-segmental sources, but not from intra-segmental neurons, were selectively depressed by muscarinic receptor activation via postsynaptic mechanisms (Mejia-Gervacio, 2012). This suggests that a level of specialization exists for ACh-induced synaptic weight modification of inputs from different sources, and that a balance between presynaptic and postsynaptic cholinergic mechanisms may participate in this process.

The first postsynaptic mechanism of ACh action on spinal motoneurons was recently discovered by Miles et al (2007). Using spinal cord slices from young mice (postnatal day 9-15), these researchers showed that bath application of muscarine induced an increase in the frequency of repetitive firing elicited by square pulse commands, observed as f/I slope increments. Oxotremorine, a muscarinic agonist, and methoctramine, a M_2 antagonist, reproduced and blocked this f/I slope increase, respectively and indicated the involvement of M_2 receptor subtype in the response to muscarine. Miles et al (2007) further showed that increments in the input-output gain of motoneurons resulted from significant reductions in AHP amplitude caused by muscarine that were, again, prevented by methoctramine. Because the AHP is essentially mediated by KCa conductances and bath applied muscarine did not alter the amplitude of calcium currents in this preparation, Miles et al (2007) inferred that muscarinic M_2 receptors mediate AHP amplitude reductions through inhibition of potassium KCa currents. It was thus concluded that ACh released from C-boutons activate M_2 muscarinic postsynaptic receptors that enhance motoneuron excitability via KCa_{SK} conductance reductions, which in turn decreases AHP amplitude. Miles et al (2007) also tested the contribution of cholinergic inputs to motoneuron output during locomotion. For this, they chemically induced locomotor-like activity in *in vitro* whole spinal cords from neonatal mice,

while the activity of groups of motoneurons was recorded extracellularly from the VRs. Local application of methoctramine via pressure ejection to one lumbar segment caused significant decreases in the amplitude of locomotor-related bursts in the corresponding VR, while physostigmine, an AChE inhibitor, increased the amplitude of these bursts. Because these drugs affected burst amplitude while rhythm frequency and coordination remained unaltered, they likely affected motoneurons but not CPG components. Miles et al (2007) concluded from these observations that cholinergic inputs enhance motoneuron output during locomotion. However, because a mix of 5-HT, NMDA, and DA was used to induce locomotor-like activity in this study, it was not determined whether ACh effects on motoneurons during motor behaviour are independent from monoaminergic influences.

The contribution of cholinergic inputs to the modulation of motoneuron output during movement was further evidenced by Zagoraiou et al (2009) studies on the C-bouton system. They generated mice lacking ChAT expression, and thus unable to release ACh, exclusively in C-boutons formed by $V0_C$ neurons and found that while locomotion remained unaffected, a 40% reduction in burst amplitude occurred during swimming in motoneurons innervating a flexor muscle, gastrocnemius. This suggested that cholinergic C-boutons enhance motoneuron excitability in a task-dependent manner and/or according to the intensity demands of muscle activation.

Studies in rodent brainstem motoneurons have shown that in addition to reducing AHP, muscarinic receptors mediate changes in other properties including V_{th} hyperpolarization, rheobase reduction and action potential shape alterations (Lape & Nistri, 2000; Nieto-Gonzalez et al, 2009; Ireland et al, 2012). In these neurons, the effect of cholinergic inputs on V_{th} is not clear and seems to depend on the type of agonist used as well

as motoneuron type. The effects of cholinergic inputs on lumbar motoneuron V_{th} are currently unknown.

RESEARCH GOALS

The overall goal of this thesis is to further characterize the control of motoneuron V_{th} by neuromodulatory systems by assessing whether the spinal cholinergic system contributes to this modulation. **More specifically, the goals of this thesis are to: (1)** determine whether ACh alters the V_{th} of mammalian lumbar motoneurons, and **(2)** assess the contribution of intraspinal cholinergic inputs to the modulation of motoneuron V_{th} during rhythmic motor output.

Paper1

To address our first goal, we performed intracellular recordings from lumbar motoneurons in spinal cords isolated from neonatal rats. To determine whether cholinergic inputs modulate V_{th}, we compared the V_{th} of motoneurons in the absence (control) and presence of different cholinergic agonists and antagonists.

Specific hypotheses:

1) **ACh induces V_{th} hyperpolarization in lumbar motoneurons**
2) **M_2 muscarinic receptors mediate changes in motoneuron V_{th} induced by ACh**
3) **Cholinergic terminals are in close apposition to the AIS of lumbar motoneurons**

The results show that although ACh can induce V_{th} hyperpolarization in quiescent networks, its modulatory actions are influenced by the physiological state of the network. M_2 muscarinic receptors as well as nicotinic receptors mediate the effects of ACh on V_{th}, and cholinergic terminals are present in close proximity to the lumbar motoneuron AIS.

Paper 2

To address our second goal, we developed a neonatal rat *in vitro* preparation to generate a rhythmic motor output occurring independently from descending influences or stimulation of lumbar segments of the spinal cord. This preparation provided a means to study and pharmacologically manipulate lumbar motoneurons during motor network activation in isolation from sensory and supraspinal input, being influenced exclusively by intraspinal systems. To determine whether propriospinal cholinergic inputs mediate changes in V_{th} during motor activity, we compared the V_{th} of lumbar motoneurons during quiescent and active rhythmic network output, prior to and following the addition of cholinergic antagonist to lumbar segments of the spinal cord.

Specific hypotheses:

1) **Intraspinally mediated state-dependent motoneuron V_{th} hyperpolarization occurs in the neonatal rat spinal cord preparation**
    - **1a) A spinally-generated rhythmic motor output can be elicited in the *in vitro* neonatal rat preparation using an approach similar to that used in the *in vivo* cat**
2) **Intraspinal cholinergic inputs contribute to the state-dependent modulation of motoneuron V_{th}**

The results show that V_{th} hyperpolarization occurs during spinally-generated rhythmic motor output in the *in vitro* neonatal rat preparation and propriospinal cholinergic mechanisms importantly contribute to this modulation.

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Chapter II.

PAPER 1:

Cholinergic modulation of the voltage threshold of neonatal rat spinal motoneurons

Edna E. Vasquez-Dominguez & Brent Fedirchuk

Abstract

Although cholinergic inputs to motoneurons represent an important modulatory source for the firing behaviour of these neurons, the physiological mechanisms underlying this modulation remain poorly characterized. The purpose of this study was to determine if acetylcholine (ACh) alters the voltage threshold for action potential initiation (V_{th}) of spinal mammalian motoneurons. Blind patch whole-cell recordings were made from antidromically identified lumbar motoneurons in isolated neonatal rat spinal cord preparations with different degrees of network intactness and activity. Bath application of ACh altered motoneuron V_{th} in state-dependent fashion, most often inducing V_{th} hyperpolarization (mean -5.5 mV) in quiescent networks and a V_{th} depolarization (mean +5.3 mV) in spontaneously active, tonically bursting networks. In both network states, ACh did not change the V_{th} of a small fraction of motoneurons (14%). Further, motoneurons in reduced spinal networks did not show changes in V_{th} at low ACh doses and V_{th} hyperpolarization was elicited only in response to high ACh doses. The effects of ACh on V_{th} were unrelated to changes in other electrical properties such as afterhyperpolarization amplitude. Enhancement of endogenously released ACh with edrophonium or activation of muscarinic receptors with muscarine reproduced the effects of exogenous ACh application on V_{th}. Further, M_2 muscarinic and nicotinic receptor antagonists reversed the effects of ACh on V_{th}, implicating both receptors subtypes in mediating ACh actions on V_{th}. Also, direct application of cholinergic antagonists altered the V_{th}, inducing mostly V_{th} depolarization and suggesting an ongoing modulatory action mediated by endogenous ACh. We conclude that the modulatory effects of ACh on motoneuron V_{th} are influenced by at least three factors, including the activity state of the network, the ACh concentration in the system and actions from other systems.

Introduction

For the production of movement, inputs from descending, intraspinal and afferent sources ultimately converge on spinal motoneurons that directly activate muscle fibres. Although motoneurons were originally thought to be a relatively passive relay of motor commands, accumulating evidence has demonstrated that their excitability is enhanced in a state-dependent fashion by neuromodulatory systems to facilitate the generation of movement. For example, lumbar motoneurons exhibit a reduction in rheobase current (Krawitz et al, 2001), a decreased afterhyperpolarization (AHP) amplitude (Brownstone et al, 1992; Schmidt, 1994), and the emergence of persistent inward currents (PICs) facilitating nonlinear integrative properties (Brownstone et al, 1994) during both fictive locomotion and scratch (Power et al, 2010) in decerebrate cats. Furthermore, the voltage threshold for action potential initiation (V_{th}) is hyperpolarized during these motor behaviours (Krawitz et al, 2001; Power et al, 2010), importantly facilitating motoneuron recruitment. A similar transient, state-dependent V_{th} hyperpolarization occurs during brainstem-evoked locomotor activity in neonatal and adult rats (Gilmore & Fedirchuk, 2004; MacDonell et al, 2015). Because this V_{th} hyperpolarization could be blocked by the 5-HT_2 antagonist ketanserin or by cooling of the cervical cord (Gilmore & Fedirchuk, 2004) and mimicked in ventral horn neurons by bath application of monoamines (Fedirchuk & Dai, 2004), descending monoaminergic systems have been suggested to mediate this effect. Our more recent work has demonstrated that state-dependent V_{th} hyperpolarization persists during fictive scratch in acutely spinal transected cats (Power et al, 2010) and during a cervically-evoked rhythmic motor output in neonatal rats in the absence of supraspinal input (Vasquez-Dominguez et al, 2010). These observations suggest that in addition to descending monoaminergic influences, an intrinsic spinal

modulatory system can also regulate motoneuron excitability. The goal of the present study is to begin the identification of this intraspinal neuromodulatory source.

Diverse modulatory systems possess the ability to alter the electrical properties of spinal motoneurons (reviewed in Renkling et al, 2000; Miles & Sillar, 2011). Among these, the spinal cholinergic system has been remarkably involved in different aspects of the locomotor function (see Jordan & Schmidt, 2002 and Miles & Sillar, 2011 for review) including adaptations following spinal cord injury (Jordan et al, 2014), and probably represents the strongest candidate for intraspinal modulation of motoneuron excitability. Although initial anatomical evidence suggested an important role of cholinergic inputs on the regulation of spinal motoneuron firing behaviour (Conradi, 1969; Conradi & Skoglund, 1969), elucidation of the physiological mechanisms underlying this modulation began only recently (for review see Frank, 2009; Witts et al, 2014).

Early reports using motoneuron recordings in cat preparations showed that iontophoretic application of acetylcholine (ACh) caused a depolarization of the membrane potential (Zieglgansberger & Reiter, 1974; Zieglgansberger & Bayerl, 1976). Although subsequent studies corroborated the idea of an overall postsynaptic excitatory effect of nicotinic and muscarinic agonists on mammalian motoneurons (Evans, 1978; Jiang & Dun, 1986; Blake et al, 1987; Newberry & Connolly, 1989; Kurihara et al, 1993; Zaninetti et al, 1999; Chamberlin et al, 2002; Liu et al, 2005; Ogier et al, 2004, 2008), no specific mechanism was suggested to explain this effect. Recently however, Miles et al (2007) reported that activation of muscarinic M_2 receptors, which are clustered at postsynaptic sites formed by cholinergic terminals called C-boutons (Muennich & Fyffe, 2004; Hellstrom et al, 2003; Wilson et al, 2004), increased lumbar motoneuron excitability by reducing a SK-type Ca^{2+}-

dependent K^+ conductance which decreases AHP amplitude. Further, whereas enhancement of endogenous cholinergic actions on motoneurons increased their output during chemically-induced locomotor-like activity, blockade of muscarinic receptor function resulted in a decrease in locomotor-related burst amplitude (Miles et al, 2007). Detailed characterization of the cholinergic cells originating the C-boutons (Zaigoraiou et al, 2009) along with the wealth of studies on this system (recently reviewed in Witts et al, 2014), have helped the understanding of how this major cholinergic input to spinal motoneurons is anatomically and functionally organized. A complete picture of the distinct conductances affected by C-boutons and other cholinergic inputs to lumbar motoneurons is still lacking however. In brainstem motoneurons for example, muscarinic agonists reduce AHP amplitude (Lape & Nistri, 2000; Nieto-Gonzalez et al, 2009; Ireland et al, 2012) along with changes in other properties including action potential shape, rheobase and V_{th} (Nieto-Gonzalez et al, 2009; Ireland et al, 2012). In hypoglossal motoneurons, muscarine but not the muscarinic agonist oxotremorine hyperpolarizes the V_{th} (Ireland et al, 2012). In contrast, carbachol, an agonist that activates both nicotinic and muscarinic receptors, produces no effects on the V_{th} of hypoglossal motoneurons (Lape & Nistri, 2000) and V_{th} hyperpolarization in oculomotor nucleus motoneurons, presumably through activation of muscarinic receptor subtypes (Nieto-Gonzalez et al, 2009).

It is not known whether the V_{th} of mammalian lumbar motoneurons is subject to modulation by cholinergic inputs. Therefore, the aim of the present study was to examine whether bath application of cholinergic agonists could alter the V_{th} of lumbar motoneurons of the neonatal rat spinal cord *in vitro*, as well as to determine the receptor subtypes mediating this modulation. Further, given that the physiological state of a network can strongly influence

the effects of neuromodulators (see Marder et al, 2014 for a recent review), we assessed whether the effects of ACh on motoneuron V_{th} were affected by the motor output as well as the integrity of the lumbar network. Additionally, we used immunohistochemical techniques to assess whether the motoneuron axon initial segment (AIS), the site where action potentials are initiated (Coombs et al, 1957a,b), receives cholinergic terminals. Portions of this work have been presented in preliminary form (Vasquez-Dominguez et al, 2011; Vasquez-Dominguez & Fedirchuk, 2012).

Methods

All surgical and experimental protocols were conducted in compliance with the guidelines for the ethical treatment of animals issued by the Canadian Council for Animal Care and approved by the University of Manitoba Animal Ethics Committee. A schematic illustration of the experimental preparation is shown in Figure 1A.

Surgical procedures

Neonatal Sprague-Dawley rats (postnatal day 1-4; n = 76) were anesthetized with isoflurane in a chamber. After decapitation, rats were eviscerated and placed in a dish filled with 4°C dissecting artificial cerebral spinal fluid (dACSF) superfused with 95% O_2-5% CO_2 for dissection. A ventral spinal laminectomy exposed the spinal roots which were cut near the dorsal root ganglia. The spinal cord was then removed, pinned to a Sylgard-lined dish, and the dura matter removed. For whole lumbar cord preparations, the T9-S1 segments were isolated whereas for reduced network preparations, the L4-L5 segments were isolated and then

hemisected to separate left from right sides. The complete lumbar cord or the hemi-cord was then transferred to a Sylgard-bottomed recording chamber and pinned ventral side up with 0.1 mm insect pins. The dACSF was then replaced with recording artificial cerebral spinal fluid (rACSF) containing synaptic blockers (described below) and allowed to slowly warm at room temperature (22-24°C). For experiments using complete lumbar cords, small incisions in the pia mater were made at L2 or L5 levels to allow electrode placement.

Intracellular recordings

Motoneurons in the L2 and L5 lumbar segments were targeted for single-cell recordings using borosilicate glass microelectrodes pulled with a Narishige PP-83 two-stage puller and filled with patch electrode solution. The filled electrodes had resistances ranging from 2.7 to 4.5 MΩ and were introduced into the ventral horn from the ventral surface of the spinal cord. A whole-cell single cell recording arrangement was obtained using the 'blind patch' technique (Blanton et al, 1989). In all experiments, the ventral root corresponding to the spinal segment containing the neuron being recorded was electrically stimulated (180-560 μA) in an attempt to activate the neuron antidromically. Only data from antidromically activated neurons, which are defined as motoneurons in the present study, are reported. A Multiclamp 700A patch-clamp amplifier, Digidata 1200 A/D converter, and pClamp 9.0 software (all from Axon Instruments) were used for data acquisition. Series resistance was monitored (usually <40 MΩ) to ensure that it did not change significantly during the recording period. Neither series resistance nor tip potentials were compensated. Data were sampled at 10 kHz.

Ventral root recordings

Ventral root recordings from the second and fifth lumbar ventral roots on both the left and right sides (lL2, lL5, rL2, rL5) were obtained using suction electrodes (A-M systems, #573000). Ventral root records were band-pass filtered (30-3000 Hz), digitized at 5 kHz and captured using a PC-based data acquisition and analysis platform with special purpose software (developed by the Spinal Cord Research Centre, University of Manitoba).

Solutions and chemicals

Extracellular solutions: The dACSF contained (in mM): NaCl (25), sucrose (88), KCl (1.9), NaH_2PO_4 (1.2), $MgSO_4$ (10), $NaHCO_3$ (26), kynurenic acid (1.5), D-glucose (25), and $CaCl_2$ (1.0). The rACSF contained (in mM): NaCl (125), KCl (2.5), $NaHCO_3$ (26), NaH_2PO_4 (1.25), D-glucose (25), $MgCl_2$ (1), and $CaCl_2$ (2.5). The pH of these solutions was adjusted to 7.3 with KOH. Osmolarity was adjusted to 305 mOsm by adding sucrose to the solution.

Synaptic blockers: In most experiments, synaptic activation was reduced by addition of the following agents to the rACSF (in µM): AP5 (10), CNQX (10), bicuculline (10) or picrotoxin (10), and strychnine (1 or 10) to block NMDA, AMPA/kainate, $GABA_A$ and glycine receptors, respectively.

Electrode solution: The patch electrode solution contained (in mM): K-gluconate (120), NaCl (5), HEPES (10), EGTA (5), $MgCl_2$ (2), $CaCl_2$ (1), Mg-ATP (5), and GTP-Na_2 (0.5). KOH was used to adjust the pH to 7.3 and osmolarity was adjusted to 305 mOsm.

Cholinergic drugs: Drug application was by means of addition to the perfusate and included the following: acetylcholine chloride (ACh, 10 – 320 µM), muscarine chloride (10 – 320 µM), edrophonium chloride (EDRO, 5 – 240 µM), methoctramine hydrate (Methoc, 10 – 60 µM),

and mecamylamine hydrochloride (Mecam, 15 – 120 µM). All drugs were obtained from Sigma and were initially dissolved in distilled water and stored as 10 mM stock solutions. To obtain the selected final bath concentration of a drug, stock solution was directly added in fractionated amounts with the use of a micropipette at the periphery of the 10 mL stationary chamber. All concentrations specified in this report, including ranges, refer to the final bath concentration. In several experiments for each drug, incremental concentrations were tested, starting with a low concentration and increasing the concentration to determine the concentration required to be effective. Washout of the drugs was accomplished by repeated exchange of the bath solution with normal solution. This was performed with the use of a syringe at a rate of 5-10 ml/min.

Measurement of V_{th}

Both, current-clamp and voltage-clamp records were included in the sample. However, most recordings (65/75) were made in voltage-clamp configuration because the V_{th} values using this technique were more stable and faster to determine than those obtained in current-clamp configuration. In current-clamp, the V_{th} for eliciting an action potential can be directly measured from the voltage record as the membrane potential at the point of maximal change of voltage (set at ≥10 V/s in the present study) at the onset of an action potential evoked by an injected depolarizing current step or ramp. V_{th} was defined as the value of membrane potential of the data point that was ≥1 mV more depolarized than the preceding point because a 10 kHz digitization rate was used throughout the present study (Power et al, 2010). In effect, this method is analogous to choosing the point at which the slope of the first derivative of membrane potential exceeds 10 V/s. In voltage-clamp mode, the set of events underlying an

action potential are observed as a fast inward current. In this configuration, an initial holding potential of -60 mV was used to approximate the resting membrane potential of the neurone, and 200-ms depolarizing pulses were successively delivered in +2 mV increments at a repetition rate of 2 Hz. Fast inward currents which would have mediated action potentials in current-clamp configuration were evident on the recorded current trace and the potential of the smallest depolarizing step capable of inducing a fast inward current was defined as the V_{th} (Fedirchuk & Dai, 2004). A hyperpolarization of V_{th} manifests as a smaller depolarizing voltage step (i.e. more negative membrane potential) being able to induce a fast inward current, whereas V_{th} depolarization is the opposite (i.e. a larger voltage step is required, and V_{th} is a less negative membrane potential). It has been previously demonstrated that V_{th} measured in voltage clamp are tightly correlated with those measured in current clamp (Dai et al, 2009). Prior to drug application, repeated trials (range = 5–12 trials; mean = 7 trials) of the protocol for measuring V_{th} ensured that the control V_{th} remained stable. Following drug administration, V_{th} was measured every 30-60 s for up to 10-40 min. Therefore, in this study each neuron served as its own control. The V_{th} data reported are averages of several trials per neuron in each condition. To ensure that a neuron's response had not been altered by a preceding drug exposure, only one neuron was recorded for each spinal cord preparation.

Measurement of other cell membrane properties

PIC amplitude: In voltage-clamp mode, PICs were elicited by a symmetrical slow voltage ramp of 10 s duration from a holding potential of -70.0 mV to a peak voltage of +10.0 mV (16.0 mV/s). The PIC evoked on the ascending phase of the voltage ramp was used and the leak current was subtracted before calculation of PIC amplitude. A straight line was drawn

along the horizontal current trace, and the average of the points prior to where the straight line is tangent to the current trace was defined as PIC baseline. An average of the lowest points on the current trace was defined as the maximal PIC amplitude. The amplitude of the PIC was calculated as the difference between the baseline and maximal PIC amplitude (Power et al, 2012).

AHP amplitude: In current-clamp mode, suprathreshold 200 ms current pulses were used to evoke action potentials prior to and after drug application. Due to the challenge of choosing an appropriate baseline from which to measure the peak amplitude of the AHP, quantitative measures of AHP are not reported in the present study. Only qualitative comparisons of the AHP under different conditions are provided here.

Input resistance (R_{in}): In current-clamp, R_{in} was measured from the changes in voltage responses to 50 ms hyperpolarizing current pulses. Similarly, R_{in} in voltage-clamp was evaluated from the current responses to 50 ms hyperpolarizing voltage steps.

Statistical analysis

Statistical analysis was performed using SigmaPlot (Systat Software Ink, version 12.5). The *chi*-square test (χ^2 test; categorical outcome variable) was used to determine associations between changes in V_{th} (V_{th} hyperpol-, depol-arization or no change) and other parameters. Pearson product moment correlation tests (Pearson correlation) were used to assess associations between the absolute values from distinct cell membrane properties or between the magnitudes of their changes. Finally, differences in membrane properties before and after pharmacological manipulations were assessed using Student's paired *t*-tests. For all tests, a $P < 0.05$ was considered significant.

Immunohistochemistry

Adult Sprague-Dawley rats (n = 2) were deeply anesthetized with a mix of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) and pre-fixed by intracardiac perfusion with 40 mL of cold (4°C) solution containing 50 mM sodium phosphate buffer, pH 7.4, 0.9% NaCl, 0.1%, sodium nitrite and 1 unit/ml of heparin. This was followed by perfusion of freshly prepared fixative solution containing cold 0.16 M sodium phosphate buffer, pH 7.1, 0.2% picric acid and 2% paraformaldehyde. Animals were then perfused with a cold solution containing 25 mM sodium phosphate buffer, pH 7.4 and 10% glucose. Spinal cords were then removed and placed in cryoprotectant solution containing 25 mM phosphate buffer, pH 7.4, 10% sucrose and 0.04% sodium azide, and stored at 4°C for 24 h. Transverse sections of lumbar segments were cut at a thickness of 14 µm using a cryostat, collected in gelatinized glass slides and stored at -20°C.

The following antibodies were used: anti-ChAT goat polyclonal (AB144P, Millipore, Temecula, CA, USA) used at a dilution of 1:100; or anti-VAChT guinea pig, polyclonal (AB1588, Millipore, Temecula, CA) at a 1:500 dilution; and anti-AnkG mouse monoclonal (33-8800, Invitrogen, Camarillo, CA) at a 1:200 dilution. Cy3-conjugated donkey anti-goat immunoglobulin G (IgG) diluted 1:300, Cy3-conjugated donkey anti-guinea pig IgG diluted 1:300, Alexa 488-conjugated donkey anti-mouse IgG diluted 1:1K, and Alexa 488-conjugated donkey anti-rabbit IgG diluted 1:500 were used as secondary antibodies (all from Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Slide-mounted sections removed from storage were air dried for 10 min, washed for 20 min in Tris-buffered saline with 0.3% triton X10 (TBSTr), and incubated simultaneously with two primary antibodies for 24 h at 4°C in TBSTr. Sections were then washed for 1 h in TBSTr

and incubated with secondary antibodies for 1.5 h at room temperature. All sections were coverslipped with antifade medium (Fluoromount-G, SouthernBiotech, Birmingham, AB, USA). Single scan images were acquired using a Zeiss Axioskop2 fluorescence microscope. Final images were processed with Adobe Photoshop CS software (Adobe Systems, San Jose, CA, USA).

Results

Effects of bath application of cholinergic agonists on motoneuron voltage threshold

ACh can hyperpolarize or depolarize the V_{th} of lumbar motoneurons

In order to assess whether cholinergic inputs to lumbar motoneurons modulate the V_{th}, we applied acetylcholine (ACh) to the bathing solution of 76 antidromically identified spinal motoneurons recorded in L2 or L5 segments. Most motoneuron recordings (70/76) were made in the presence of synaptic blockers in the bathing ACSF. Initial experiments showed the absence of significant differences between the change in V_{th} recorded in solution with or without the presence of synaptic blockers (χ^2; n = 12, P = 0.55). Consequently, all motoneurons were grouped together for further analysis. Synaptic blockers were used to synaptically isolate the recorded motoneuron and thus assess the postsynaptic effects of ACh. The membrane resistance and access resistance were monitored constantly throughout the course of each experiment. The absolute value of V_{th} determined using the voltage- and current-clamp protocols ranged from -20.7 to -58.0 mV (mean = -38.3 ± 7.5 mV). There was no relation between the motoneuron R_{in} (range = 106.2 to 628.3 MΩ) and its absolute V_{th} (Pearson correlation; n = 68, P = 0.63).

ACh elicited a change in the V_{th} of 65/76 motoneurons (86%). ACh could elicit V_{th} hyperpolarization in some motoneurons and V_{th} depolarization in others. Figure 1 illustrates representative examples of the effect of ACh on the V_{th} of two different lumbar motoneurons. Figure 1A shows a schematic illustration of the experimental preparation in which the lumbar spinal cord of neonatal rats (left) was isolated for network and individual motoneuron recording (right). For the L5 motoneuron in Figure 1B, a step to -40.0 mV was required to elicit the first inward current (Control, left) and was its V_{th}. Within 2 minutes of the addition of 30 µM ACh to the bathing solution, the first fast inward current was elicited by a smaller depolarizing step, to -44.0 mV (ACh, red trace). This difference represents a -4.0 mV hyperpolarization of the V_{th} and it was reversed by washing out the ACh. As illustrated in Figure 1C, ACh was also able to induce reversible V_{th} depolarization (blue trace) in a different motoneuron. A change from -30.0 to -24.0 mV following ACh application was observed in this example. The effect of ACh on the V_{th} from all motoneurons tested is summarized in Figure 2. ACh induced V_{th} depolarization in 31/76 (41%) motoneurons (blue bars) and the magnitude of change was +2.0 to +14.0 mV (mean = +5.3 ± 2.7 mV). 34/76 (45%) motoneurons showed V_{th} hyperpolarization in response to ACh (red bars) and the magnitude of change was -2.0 to -24.9 mV (mean = -5.5 ± 4.7 mV). Finally, 11/76 (14%) motoneurons did not change their V_{th} after ACh application (gray bars).

Incremental increases in ACh concentration (10-130 µM) showed that 35 µM ACh caused a change in V_{th} in 50% of cases and that increasing doses did not result in increasingly larger V_{th} changes (data not shown; n = 60). This impeded the construction of a dose-effect curve. The effects of ACh on V_{th} occurred within 2-14 min and could occur in the absence of any discernible motor output. Washout of the effects of ACh was attempted in 17 experiments

from which the V_{th} recovered to values close to those of control in 5/17. From these, V_{th} returned to the exact control values (2/5), or recovered to values within ± 4.0 mV respect to control values (3/5) after the wash.

Whether ACh depolarized, hyperpolarized or did not affect V_{th} was not correlated with: initial V_{th}, postnatal age of the preparation, segmental location of the motoneuron (L2 or L5), cell total capacitance, amplitude of the first inward current, or presence of detectable PICs (χ^2 tests; all $P > 0.05$). Also, effects on V_{th} were not associated with changes in access resistance during the recording (χ^2 test; $n = 60$, $P = 0.19$).

The effect of ACh on V_{th} is independent from ACh's effects in other membrane properties

We assessed whether in addition to V_{th}, ACh modulated other electrical properties influencing motoneuron excitability and whether changes in these parameters were correlated with the effects on V_{th}. There was no correlation with the change of V_{th} and the initial R_{in} of the neuron, however the R_{in} of 38/68 motoneurons was significantly reduced during ACh administration (paired *t*-test; $P < 0.001$). Using an arbitrary value of 5% as a minimum significant reduction in R_{in}, the mean percent decrease induced by ACh was 22% and ranged from 5 – 61%. There was no relation between the magnitude of V_{th} change and R_{in} change (Pearson correlation; $P = 0.20$). In 42/65 neurons, the amplitude of the fast inward current evoked by depolarizing steps was significantly decreased (paired *t*-test; $P < 0.001$) following ACh application to the bath. Again using 5% as the minimum reduction in current amplitude, the mean percent decrease was 30% and ranged from 6 – 81%. There was no relation between the magnitude of inward current amplitude reduction and V_{th} change (Pearson correlation; $P = 0.33$).

Assessment for the presence of PICs was performed by symmetrical slow voltage ramps (see Methods for details on PIC amplitude measurement) in 52 motoneurons. In control conditions only 13/52 motoneurons showed clear presence of PICs (mean peak amplitude = 40.1 ± 19.3 pA; range = 18.2 – 98.5 pA). The total peak amplitude of PICs was significantly reduced after addition of ACh to the bath in 10/13 neurons (mean % reduction = 36%, range = 8 – 79% decrease). There was no relation between the magnitude of V_{th} change and PIC amplitude change (Pearson correlation; P = 0.19).

Motoneurons recorded in current clamp mode (n = 12) were used for measurement of resting membrane potential (resting V_m) and AHP amplitude. In 8/12 neurons ACh produced a modest depolarization of the resting V_m (mean = +6.0 ± 4.4 mV) whereas in the other 4 neurons the resting V_m did not change after ACh. AHP amplitude was assessed by initiating an action potential using 200 ms current steps. Others (Brownstone et al, 1992; Miles et al, 2007; Ireland et al, 2012) have used the V_{th} or the resting V_m as baseline when measuring the AHP; however this would over or under-estimate the AHP because ACh can change the V_{th} or alter the resting V_m, therefore we report the qualitative change in AHP induced by ACh. ACh caused a reduction in the AHP amplitude in only 3/12 motoneurons; it increased AHP amplitude in 4/12 motoneurons and did not change AHP amplitude in 5/12 motoneurons. As illustrated in Figure 3, ACh could affect V_{th} and AHP peak amplitude independently. ACh-induced V_{th} hyperpolarization could be associated with either an AHP amplitude increase, decrease or no change, and the same was true for ACh-induced V_{th} depolarization. Accordingly, there was no relation between the effect of ACh on V_{th} and AHP (χ^2 test, P = 0.65).

The effect of ACh on motoneuron V_{th} is influenced by network activity

In experiments in which a complete lumbar cord was used to study motoneuron V_{th}, the overall network output was monitored though suction electrodes on the L2 and L5 ventral roots on both sides of the cord (Figure 1A). Prior to ACh application, motor network quiescence reflected by the lack of bursting activity in all four ventral roots was observed in 60% of experiments (45/76) whereas in the remaining 40% (31/76) spontaneous network activity was evident. This activity consisted of bursting synchronous among two or more ventral roots in 20/31 experiments (65%), tonic spiking in one or two ventral roots in 9/31 (29%), or rhythmic bursting in one or more ventral roots in 2/31 (6%) experiments. A tendency of ACh to have differential effects on motoneuron V_{th} depending on the presence or absence of network activity was found (Figure 4). Figure 4A-B shows a representative experiment in which the network remained quiescent both before and after bath application of ACh, whereas the V_{th} of the simultaneously recorded motoneuron was hyperpolarized by -4.0 mV (red line) after ACh administration. Figure 4C-D shows a different experiment in which the lumbar network was spontaneously active and ACh-induced V_{th} depolarization (blue line) was observed in the corresponding motoneuron. In both examples of Figure 4, synaptic blockers were present in the solution bathing the spinal cord. Graph in 4E summarizes the proportion of the different effects of ACh on motoneuron V_{th} in two groups of experiments defined by the presence or absence of spontaneous ventral root activity. In spinal cord preparations with a quiescent network, the number of motoneurons showing V_{th} hyperpolarization after ACh (24/45; 53%) was larger than the number of motoneurons showing V_{th} depolarization (15/45; 33%). On the contrary, in preparations with an active network this proportion was inversed: 10/31 motoneurons (32%) displayed V_{th}

hyperpolarization whereas 16/31 motoneurons (51%) displayed V_{th} depolarization. The proportion of neurons that did not show a change in V_{th} after ACh remained similar in both groups of spinal cords (13 and 16% in preparations with quiescent and active networks, respectively). The tendency toward V_{th} depolarization in active networks was not related to whether the motor output consisted in synchronous, tonic or rhythmic activity. The trend for motoneurons in quiescent networks to show a hyperpolarization of their V_{th} and motoneurons in active networks to have a depolarization of their V_{th} after ACh, however, was not statistically significant (χ^2 test, P = 0.18). Nevertheless, this observation suggests that ACh may modulate motoneuron excitability according to the state of the network.

ACh application did not induce ventral root activity or alter the existing network activity in most experiments (57/76; 75%). ACh induced rhythmic bursting in only 3/76 experiments, with a pattern similar to that described by Cowley & Schmidt (1994). In those experiments in which ACh altered the existing network activity (19/76; 25%), it either increased (11/19; 58%) or decreased (8/19; 42%) the intensity of this activity (e.g. burst amplitude and number of bursts occurring per minute). As the example illustrated in Figure 4C-D, in those experiments in which the network output decreased after ACh, most neurons (5/8; 63%) displayed V_{th} depolarization, consistent with an overall depressive effect of ACh in these preparations. Analysis of the effects of ACh at the motoneuron and network levels confirmed a statistically significant tendency of ACh to depolarize V_{th} in networks in which this drug concomitantly reduced ventral root output intensity (χ^2 test; n = 76, P = 0.03). ACh affected the network prior to affecting individual motoneuron V_{th} in most cases in which this drug affected both parameters (9/13). This observation was unrelated to the direction of the effects of ACh on V_{th} or ventral root activity.

The effects of other cholinergic drugs on motoneuron V_{th} are similar to ACh effects

In order to examine whether the effects of bath applied ACh on motoneuron V_{th} were mimicked by activation of muscarinic receptors or by endogenous cholinergic actions, muscarine (a non-selective muscarinic agonist) and edrophonium (EDRO; an acetylcholinesterase inhibitor) were used, respectively. Similar to ACh, bath-applied muscarine (10-50 μM) induced V_{th} hyperpolarization in 6/10 (mean = -3.3 ± 1.8 mV), V_{th} depolarization in 3/10 (mean = +5.6 ± 3.8 mV) and no change in V_{th} in 1/10 motoneurons (Figure 5B). Changes in V_{th} could occur in the absence of network activity; however, in contrast to ACh, muscarine induced rhythmic ventral root output in all preparations in which this agent was tested (n = 10). This rhythmic motor output, although similar to locomotor-like activity at some periods, consisted of 0.2-0.5 Hz bursting that lacked consistent L2-L5 or left-right alternation (Figure 5A). Muscarine induced rhythmic activity even at low doses (10 μM) and in spite the presence of the synaptic blockers within the bathing ACSF. The effects of muscarine on both motoneuron V_{th} and network output were reversed after washout in 3 of 4 attempts.

In 8 experiments, EDRO (5-240 μM) was applied to the bath alone and after 8-20 minutes ACh was added. Quickly after EDRO was applied alone, it elicited a single synchronous ventral root burst followed by quiescence (Figure 5C). Associated with this burst, transient motoneuron V_{th} hyperpolarization was observed in 5/8 experiments (Figure 5D; mean = -5.0 ± 3.7 mV). The V_{th} values of each neuron returned to those in control conditions after the EDRO-induced burst had ended. Addition of ACh to the bath after this event resulted in the generation of another synchronous burst followed by sustained bursting activity that lacked a clear pattern or rhythm (not illustrated). Relatively stable V_{th}

hyperpolarization was observed after EDRO and ACh were both present in the bath. The magnitude of hyperpolarization after ACh was similar to that produced by EDRO for each neuron (mean = -4.2 ± 3.5 mV). EDRO alone or in combination with ACh produced V_{th} depolarization in 3 experiments (Figure 5D; mean = +6.0 ± 4.0 mV). Interestingly, one of these three cases was singular in that synchronous ventral root activity was present previous to drug application. Neither EDRO nor ACh elicited the minute-lasting burst in the ventral roots, and only stable motoneuron V_{th} depolarization was observed in this experiment.

The effect of exogenously applied ACh on motoneuron V_{th} is sensitive to the endogenous cholinergic network

Because the lumbar network activity state seemed to influence the effect of the bath-applied ACh on motoneuron V_{th}, we hypothesized that assessment of V_{th} in preparations with a reduced network would allow determination of a more direct effect of ACh on individual motoneurons. For this purpose, hemi-sectioned spinal cords containing only two lumbar segments (L4 and L5) were used for recording of the effects of bath application of ACh (30-320 µM) on motoneuron V_{th} (Figure 6A). ACh in this 'reduced network' spinal cord preparation induced V_{th} hyperpolarization in 6/16 (38%) neurons, V_{th} depolarization in 3/16 (19%) neurons and no change in V_{th} in 7/16 neurons (44%) (Figure 6B). The magnitude of the V_{th} change was -4.0 to -8.0 mV (mean = -5.0 ± 1.6 mV) for V_{th} hyperpolarization and +1.9 to +6.0 mV (mean = +3.3 ± 2.3 mV) for V_{th} depolarization. As for previous experiments, the outcome in V_{th} was not dependent on the presence of synaptic blockers in the bath (χ^2 test; n = 8, P = 0.29), and it could be reversed with washout (attempted in one experiment only). The network output monitored through L4 and L5 ventral roots showed sustained quiescence both

before and after ACh administration in all hemi-cord preparations. These results are similar to those using whole lumbar spinal cords with a quiescent network in that the proportion of ACh-induced V_{th} hyperpolarization is larger than the V_{th} depolarization. However, these had a proportion of neurons with a V_{th} unresponsive to ACh that was much larger than experiments using whole lumbar spinal cords (44% and 14%, respectively).

Similar to complete lumbar cord preparations, only the initial doses of ACh applied within the first 15-20 min determined whether a change in motoneuron V_{th} occurred or not, and increasing doses of ACh after this period did not result in increasing changes in V_{th} (or increased probabilities to elicit a V_{th} change where there was previously none). We assessed whether initial low doses (30-60 µM) of ACh differed from initial high doses (100-320 µM) in their ability to alter motoneuron V_{th} in the reduced network preparation. It was observed that the percentage of neurons with a V_{th} unresponsive to ACh was slightly larger in experiments using initial low doses than experiments using initial high doses (50% and 37%, respectively). Further, when initial high doses were used, all but one of the responsive neurons showed V_{th} hyperpolarization (4/5, 80%). The observation that larger doses of exogenously applied ACh are required to increment the probability of this drug to modify motoneuron V_{th} in preparations in which the cholinergic network surrounding the motoneuron is severely reduced, suggests that a cooperative effect exists between exogenous and endogenous sources of ACh. The presence of spontaneous release of endogenous ACh in *in vitro* spinal cord preparations is also suggested by experiments using EDRO (see previous section) as well as experiments using direct application of cholinergic antagonists (see next section).

Receptor subtypes mediating ACh-induced changes in motoneuron V_{th}

According to results in this study, ACh could induce lumbar motoneuron V_{th} hyperpolarization or depolarization. Our next aim was then to identify the receptor subtypes mediating these changes. We started by examining whether cholinergic antagonists alone had direct effects on the V_{th} of lumbar motoneurons. Later, we assessed the ability of these antagonists to block the effects of ACh on V_{th}.

Blockage of endogenous muscarinic or nicotinic receptor activity affects motoneuron V_{th} and network activity

The M_2 receptor antagonist methoctramine (Methoc) was tested in its ability to directly modulate lumbar motoneuron V_{th}. Methoc (10-60 μM) induced V_{th} hyperpolarization in 5/11 (45%), V_{th} depolarization in 5/11 (45%) and no change in V_{th} in 1/11 (9%) neurons (Figure 7A). The mean V_{th} change was -5.1 ± 3.3 mV and +6.7 ± 4.1 mV for hyperpolarization and depolarization, respectively. In the majority of these experiments (9/11), spontaneous synchronous activity was present and it was reduced to a state of quiescence in 6/9 cases after Methoc. A recovery of V_{th} close to control values and reappearance of spontaneous network activity was observed after addition of ACh to preparations in which Methoc initially induced V_{th} depolarization (4/4), demonstrating ACh's ability to counteract the effects of Methoc. This ability was not observed in preparations in which Methoc induced V_{th} hyperpolarization. Washout of the effects of Methoc alone or in combination with ACh was successful in 2/3 attempts.

The nicotinic antagonist mecamylamine (Mecam; 15-120 μM) induced V_{th} hyperpolarization in 2/9 (22%; mean = -4.0 ± 0 mV), V_{th} depolarization in 5/9 (55%; mean = +10.3 ± 8.0 mV) and no change in V_{th} in 2/9 (22%) motoneurons (Figure 6B). The tendency of Mecam to induce motoneuron V_{th} depolarization was unrelated to the activity state of the network. Even though Mecam could either increase (3/9), decrease (3/9) or not affect (3/9) network output, it produced a transient period of quiescence in all cases (9/9). Addition of ACh to an existing effect of Mecam did not produce further changes in V_{th}, although it tended to increase the ventral root activity. Washout of the effects of Mecam alone or in combination with ACh was successful in 1/3 attempts.

It was concluded from this data that endogenous cholinergic inputs regulate lumbar motoneuron V_{th} in basal conditions. This is reflected by changes in V_{th} after blockage of muscarinic and nicotinic receptors, which together consisted mostly in V_{th} depolarization. The effects of cholinergic antagonists on V_{th} appear to be unrelated to the activity state of the network, despite the antagonists ability to depress motor activity.

Nicotinic and M_2 muscarinic receptors mediate ACh-induced changes in motoneuron V_{th}

Next we tested the ability of Methoc and Mecam to block the changes in motoneuron V_{th} induced by ACh. For this, ACh was initially applied to the bath and once a stable change in V_{th} was detected the antagonist was added in increasing doses to assess whether it reversed the V_{th} to values close to the control V_{th}.

Involvement of M_2 muscarinic receptors in the V_{th} changes induced by ACh

Methoc (10-50 µM) showed an ability to revert ACh-induced V_{th} hyperpolarization (10/13; 77%) and ACh-induced V_{th} depolarization (6/11; 55%) (Figure 8A-B, coloured lines). V_{th} values after Methoc could be equal, slightly positive or slightly negative compared to control values. The ability of Methoc to revert ACh-induced changes in motoneuron V_{th} was not contingent on the activity state of the network or the effect of ACh on this activity. Further, although ventral root output after Methoc could either increase (13/24; 51%) or remain the same (11/24; 46%), this effect was not related to its ability to block ACh-induced changes in V_{th}. The additive effects of ACh and Methoc on motoneuron V_{th} were reversed after washout in 2/6 attempts.

As described above, ACh reduced the R_{in} of most motoneurons in this study. Methoc produced a recovery of R_{in} in 11/17 neurons (65%), to values below (5/11) or above (6/11) the original R_{in}. Interestingly, in all neurons in which Methoc reversed the ACh-induced V_{th} hyperpolarization, this antagonist reversed the R_{in} reduction as well (6/6). In contrast, none of the neurons in which Methoc was able to block the ACh-induced V_{th} depolarization displayed a recovery in the R_{in} reduction (0/2). Opposite to R_{in}, the ACh-induced reductions in the amplitude of fast inward currents and PICs were much less susceptible to being reversed by Methoc (only 4/20; 20% and 1/4; 25%, respectively). The effects of ACh on AHP amplitude, consisting of an increase in 2/3 neurons and decrease in 1/3 neurons, were all partially reversed by Methoc (30-56% recovery; see e.g. Figure 3). This ability of Methoc to reverse the AHP changes was unrelated to its ability to reverse changes in V_{th}.

Involvement of nicotinic receptors in the V_{th} changes induced by ACh

Although the effect did not occur as frequently as that seen during M_2 receptor activation, nicotinic receptors also seemed to be able to mediate V_{th} changes induced by ACh. Figure 8C-D shows that Mecam (10-80 μM) reversed ACh-induced V_{th} hyperpolarization in 6/9 neurons (67%) whereas it failed to reverse V_{th} depolarization in most cases (reversal occurred in 1/8; 1%). Similar to the results for Methoc, V_{th} after Mecam recovered to values equal or slightly different than control values, and this ability was not related to the network output prior to drug application, the effect of ACh on ventral root activity or the activity state of the network after Mecam. Mecam reversed ACh-induced R_{in} decreases in most motoneurons tested (9/12; 75%) independently of whether it additionally reversed changes in V_{th}. The R_{in} following the addition of Mecam was usually lager than that of control values (8/9 neurons). Reductions in fast inward current, PIC and AHP amplitude induced by ACh were seldom affected by Mecam (5/16, 0/2 and 0/3 neurons, respectively). Therefore although both M_2 and nicotinic receptors are able to mediate changes in motoneuron excitability by modification of the V_{th} and other properties, muscarinic M_2 receptors seem to be more strongly mediating ACh effects.

Anatomical evidence of cholinergic modulation of motoneuron V_{th}

Cholinergic inputs are in close apposition to the axon initial segment of some lumbar motoneurons

We tested the hypothesis that cholinergic modulation of motoneuron V_{th} is exerted through direct inputs in the AIS of these neurons. For this, cholinergic terminals were identified with anti-ChAT or anti-VAChT antibodies that label the choline acetyltransferase

enzyme and the vesicular acetylcholine transporter, respectively (Barber et al, 1984; Arvidsson et al, 1997). An antibody directed against Ankyrin G, a scaffolding protein expressed throughout all the AIS (Kordeli et al, 1995), was used to label the motoneuronal AIS. Preliminary results in adult rats show that cholinergic terminals are in close proximity to the AIS of some lumbar motoneurons. Some examples are shown in the single-scan wide-field images in Figure 9. Figure 9A shows VAChT (A1, red) and AnkG (A2, green) staining in lamina VIII L4 motoneurons. Overlay of these sections (A3) reveals VAChT-positive terminals in proximity to the soma and dendrites of a motoneuron as well as in close association with its AIS (arrow). Other motoneurons in both L4 and L2 segments of the cord also show a clear apposition of cholinergic terminals along the AIS length (Figure 9B-C, arrows). In some motoneurons, however, there were no obvious associations between cholinergic terminals and AIS, as the example in Figure 9D. Although detailed quantitative analysis has not yet been performed, the presence of cholinergic inputs in close proximity to AIS of lumbar motoneurons was not an infrequent finding; approximately one to two examples could be observed per every couple of subsequent 14 µM transversal sections of spinal cord tissue. Further analysis and additional studies are needed to more fully characterize the association between these cholinergic inputs and the motoneuronal AIS.

Discussion

Results in the present report indicate that cholinergic inputs modulate the V_{th} for action potential initiation of lumbar motoneurons according to the activity state of the motor network. Whereas acetylcholine mostly induced V_{th} hyperpolarization in quiescent lumbar

networks, V_{th} depolarization was mostly observed in response to this modulator in spontaneously active, synchronously bursting networks. Changes in V_{th} were not related to alterations in R_{in}, AHP amplitude or PICs, and were mediated by both muscarinic and nicotinic receptor subtypes.

However, all of the pharmacological agents used in this study generated highly variable effects on V_{th}. We currently have no explanation for this observation other than inter-individual differences conferring functional flexibility and adaptability (see for example Spitzer et al, 2008 and Marder & Goaillard, 2006; Harris-Warrick & Johnson, 2010; Marder, 2011; Hamood & Marder, 2015 for review). Interestingly, tendencies in drug effect direction emerged from analyses of the influence of some factors, such as network activity, on drug action. A schematic summary of the combined effects of network state and acetylcholine receptor activity on motoneuron V_{th} as observed in the present study as well as data from our study of the role of cholinergic inputs on rhythmically active networks (Vasquez-Dominguez & Fedirchuk, 2012) is illustrated in Figure 10.

Acetylcholine modulates the V_{th} of spinal motoneurons

The present results show that pharmacological manipulation of the activity of cholinergic receptors produces alterations in lumbar motoneuron V_{th}. In spinal cords with an intact or reduced network and absent activity, application of ACh tended to induce V_{th} hyperpolarization (Figs. 4E and 6; Fig. 10A-B, bright red neurons), supporting an excitatory role of ACh on motoneuron excitability. Further, blockade of endogenous M_2 muscarinic or nicotinic receptor activity produced mainly V_{th} depolarization (Fig. 7; Fig. 10B, blue neuron) whereas enhancement of endogenously released ACh mostly produced V_{th} hyperpolarization

(Fig. 5C-D; Fig. 10B, bottom bright red neuron). This suggests that basal activity of cholinergic receptors sets motoneuron V_{th} towards relatively hyperpolarized values (Fig. 10B, light red neurons).

It should be noted that bath applied ACh in the present study resulted in a similar degree of motoneuron V_{th} hyperpolarization (mean -5.5 mV) as observed during locomotion and scratch in the cat (Krawitz et al, 2001; Power et al, 2010) as well as during rat locomotion (Gilmore and Fedirchuk, 2004; MacDonell et al, 2015). Therefore, it is possible that the V_{th} hyperpolarization that occurs during fictive scratch in cervically transected cats reported by Power et al (2010) may be mediated by cholinergic mechanisms. We have developed an *in vitro* preparation to test the hypothesis that intraspinal cholinergic inputs mediate state-dependent changes in motoneuron V_{th} independently of descending influences, and found that endogenously released ACh modulates V_{th} during rhythmic motor output in preparations devoid of supraspinal structures (Vasquez-Dominguez et al, 2010; Vasquez-Dominguez & Fedirchuk, 2012). This was reflected by cholinergic antagonist-induced blockage of phasic and tonic V_{th} hyperpolarization associated with the production of rhythmic motor output (Fig. 10C, blue neurons).

Induction of V_{th} hyperpolarization by cholinergic agonists has been observed in some brainstem motoneurons (Nieto-Gonzalez et al, 2009; Ireland et al, 2012). In hypoglossal motoneurons, muscarine induces V_{th} hyperpolarization whereas the M_3-preferring antagonist 4-DAMP depolarizes the V_{th} (Ireland et al, 2012). In contrast, oxotremorine and carbachol do not alter the V_{th} of these neurons (Ireland et al, 2012; Lape & Nistri, 2000). Further, carbachol was shown to facilitate firing of oculomotor nucleus motoneurons, an effect blocked by atropine and mimicked by muscarine, implicating muscarinic receptor subtypes (Nieto-

Gonzalez et al, 2009). Although not directly measured, it is possible that V_{th} hyperpolarization contributed to these observations. The ACh-induced motoneuron V_{th} hyperpolarization observed in the present study was reversed by Methoc and Mecam in 77% and 67% cases (Fig. 8A,C), indicating the involvement of both muscarinic and nicotinic receptor subtypes. Although nicotinic receptors have been involved in mediating an inward current depolarizing motoneurons (Blake et al, 1987; Zaninetti et al, 1999; Wang et al, 1991; Robinson et al, 2002; Ogier et al, 2004, 2006; Liu et al, 2005; Mine et al, 2015), their effect on V_{th} remained unexplored. The present results are the first to show that nicotinic receptors, although involved in a lesser extent than muscarinic receptors, mediate changes in spinal motoneuron V_{th}. Together, this evidence suggests that cholinergic contributions to the modulation of motoneuron V_{th} and the receptor subtypes involved might vary among motoneurons with different functions and cholinergic input sources.

Distinction between presynaptic and postsynaptic effects of ACh on motoneuron V_{th} was not possible in this study. We aimed to synaptically isolate motoneurons under recording by using blockers of fast excitatory and inhibitory transmission in the bathing solution. However, 40% of spinal cord preparations displayed spontaneous network activity and this occurred in spite higher concentrations of synaptic blockers were used in an attempt to reduce this activity (data not shown). Nevertheless, the presence of cholinergic inputs that are in close apposition to the AIS of many of the adult rat motoneurons examined (Fig. 9) suggests direct postsynaptic effects on spinal motoneurons are likely the major contributor to the results of the present study. The AIS is known as the site of action potential initiation due to its low V_{th} (Coombs et al, 1957a,b; see Dulla & Huguenard, 2009; Kress & Mennerick, 2009 for review). In an electron microscopy study of cat spinal motoneurons, Kellerth et al (1979) reported the

presence of several types of synaptic endings including C-boutons on the axon hillock, suggesting that inputs on this structure exist to directly impact the V_{th}. Although our data is preliminary, the presence of VAChT-positive terminals on lumbar motoneuron AIS is consistent with Kellerth et al (1979) observations as well as our physiological data demonstrating cholinergic modulation of neonatal rat motoneuron V_{th}. Direct modulation of V_{th} by AIS 5-HT_{1A} receptor-induced modulation of Na^+ currents, has been demonstrated in turtle spinal motoneurons, albeit via serotoninergic spillover mechanisms (Cotel et al, 2013).

State-dependent modulatory effects of ACh on motoneuron V_{th}

One of the main findings of the present study was that the effects of ACh on motoneuron V_{th} were influenced by the physiological state of the lumbar network (Fig. 10). In networks with a quiescent motor output, the proportion of neurons displaying V_{th} hyperpolarization in response to ACh was higher than neurons displaying V_{th} depolarization (53% and 33%, respectively; Fig. 4E; Fig. 10B, top bright red neuron). The opposite proportion in the direction of the effect of ACh on V_{th} was observed in preparations with spontaneously active networks (Fig. 10D, top blue neuron). Further, preparations with a reduced network (e.g. 2 segment-long hemi-cords) showed a proportion of ACh effects on V_{th} as follows: no change in V_{th} (44%) > V_{th} hyperpolarization (38%) > V_{th} depolarization (19%) (Fig. 6). These observations indicate that the modulatory effects of ACh on motoneuron V_{th} are sensitive to both the activity state and the integrity of the network.

A growing number of physiological and computational studies highlight that individual neuron and network responses are a function of network states (see Nadim et al, 2008 and Marder et al, 2014 for review). Differences in the activity state of a network, prior to

any experimental intervention, have shown to explain discrepancies in the direction of the effects of a neuromodulator in invertebrate systems (see for example Nusbaum & Marder, 1989; Skiebe & Schneider, 1994; Weimann et al, 1997; Ma et al, 2009; Szabo et al, 2011). Network activity can influence modulatory effects on target neurons by activation of voltage-dependent conductances via increases in stochastic synaptic activity (Wolfart et al, 2005; Nadim et al, 2008), or by activation of second-messenger transduction pathways that crosstalk with those activated by a modulator (Yu et al, 2004; Marder et al, 2014). In some cases, inconsistent responses to a modulator do not depend on an obvious parameter of the network state but are the consequence of inter-individual variability in the underlying neuron or circuit features (see for example Spitzer et al, 2008; Grashow et al, 2009). Further, the state of a network can in turn be modified by modulators and thus the state-dependent effect of a modulator may potentially result from meta-modulation processes (see for example Svensson et al, 2001). Interestingly, our observation that ACh can induce V_{th} hyperpol- and depol-arization according to the network state of neonatal rats, raises the intriguing possibility that, in high spinal adult cats in which the V_{th} of motoneurons from separate limbs displays opposing changes during scratch (Power et al, 2010), a single spinal cholinergic modulatory system might exert a differential (and opposite) control on contralateral limbs.

In our scheme in Fig. 10, we considered 3 factors influencing the actions of ACh on motoneuron V_{th}: network activity state, ACh concentration (or cholinergic receptor activity) and other systems actions. Sensitivity to ACh concentration was evidenced by our experiments using low and high ACh doses on reduced network preparations. Whereas low doses generally induced no change in V_{th}, high doses elicited V_{th} hyperpolarization in the 50% of neurons that responded (Fig. 10A). This suggests that a concentration threshold exists for

ACh to alter motoneuron V_{th}. Similar to ACh concentration, the degree to which other systems influenced ACh effects on V_{th} (yellow background) was suggested by contrasting preparations with quiescent and tonically active networks (Fig. 10 B *vs* D). Network hyperexcitability is associated with the generation of the spontaneous activity characteristically observed in immature spinal cords from chicks and rodents (O'Donovan, 1999). The observation that ACh tends to hyperpolarize motoneuron V_{th} in networks with low excitability levels (e.g. with quiescent motor output) compared with hyperexcitable networks (e.g. with spontaneous tonic motor output) suggests that regulatory mechanisms exist so that the enhancement of motoneuron excitability exerted by ACh is limited in hyperexcitable networks, preventing the system from over-excitation. Supporting this notion, and although failing to meet statistical significance, the mean initial V_{th} values from motoneurons in active networks tended to be slightly hyperpolarized compared with the mean V_{th} from motoneurons in quiescent networks (-40.0 and -38.4 mV, respectively). Analogous to the 5-HT-mediated central fatigue mechanisms suggested by Cotel et al (2013), the V_{th} depolarization observed following application of ACh in spontaneously active networks may thus reflect opposing mechanisms acting to limit the activation of motoneurons in these conditions. Activity-dependent ACh-induced V_{th} depolarization may potentially result from the interaction between conductances, second-messenger pathways or modulators activated by the network and the applied ACh. We thus suggest that ACh induces V_{th} hyperpolarization in conditions where moderate levels of network activity and ACh concentration are present; deviations from these conditions (e.g. low or high levels of network activity and/or ACh release) result in V_{th} depolarization potentially caused by ineffective or excessive ACh concentrations and/or by influences from other systems (Fig. 10, bottom colored arrow).

It is noteworthy that spontaneous network activity was evident in a considerable proportion of the preparations (40%), despite the presence of synaptic blockers in the extracellular solution. This activity either illustrates the persistence of synaptic transmission, or the participation of other mechanisms (e.g. gap junction connectivity) in mediating this network output. Considering the cholinergic system was the focus of study, cholinergic antagonists were not routinely included in the mix of synaptic blockers used, and therefore ACh transmission may have contributed to the spontaneous activity observed. As described in the results, in those preparations where Methoc was applied, the spontaneous network activity was blocked in most cases (6/9). Mecam also blocked spontaneous activity (9/9 preparations), albeit the blockade was usually transient, lasting between 15-30 min before the activity resumed. Gap junctions have been implicated in mediating spontaneous activity in the developing rodent spinal cord (Hanson & Landmesser, 2003) as well as in other systems (see Roerig & Feller, 2000 for review), so it would not be surprising if they also contributed to network activity in the present experiments, perhaps particularly following synaptic blockade.

Putative mechanisms of cholinergic modulation of V_{th}

In a model developed to identify the ionic bases of the motoneuron V_{th} hyperpolarization that occurs during cat fictive locomotion, Dai et al (2002) concluded that selective modulation of Na^+ or K^+ conductances within the AIS were the most plausible mechanisms underlying this change. Supporting this model, Power et al (2012) showed that negative shifts in the activation voltage of Na^+ channels induced by a plant alkaloid neurotoxin consistently induces V_{th} hyperpolarization in neonatal rat lumbar motoneurons. Alternatively, modulation of K^+ channels of the Kv7 family mediating a subthreshold current

typically inhibited by muscarinic receptor activation (M-current; Brown & Passmore, 2009) can potentially underlie changes in V_{th} as shown in hippocampal neurons (Shah et al, 2007). The M-channel gene KCNQ2 is uniformly expressed along the AIS of mouse motoneurons (Duflocq et al, 2011). Further, M-channel inactivation with the antagonist XE991 has been shown to increase neonatal rat motoneuron excitability as observed by increases in the number of spikes elicited by stimulation of the ventral commissure (Bertrand & Cazalets, 2011). Future experiments are needed to determine whether the changes in V_{th} induced by ACh, as well as other modulators (Fedirchuk & Dai, 2004; Han et al, 2007; Tartas et al, 2010; Iwagaki & Miles, 2011), target Na^+ and/or K^+ conductances at the AIS of rodent motoneurons.

Miles et al (2007) showed that activation of M_2 receptors postsynaptic to cholinergic C-boutons on lumbar motoneurons leads to decreases in AHP amplitude by reducing a Ca^{2+}-dependent K^+ conductance. Results in the present study suggest that, independently of their effects on AHP, M_2 receptors additionally mediate changes in neonatal rat motoneuron V_{th} (Fig. 3). Whereas Miles et al (2007) reported a consistent reduction in AHP amplitude in response to muscarine in motoneurons in spinal cord slices from young mice, in neonatal rat motoneurons from whole lumbar spinal cord preparations we observed inconsistent changes in AHP following ACh application, usually no change in AHP amplitude. Although these differences might be due to species, developmental or network integrity differences, what is relevant from these observations is that cholinergic modulation of motoneuron excitability may be exerted via independent mechanisms mediated by identical receptor subtypes. The independence between AHP and V_{th} modulation is suggested by electrophysiological (Power et al, 2010) and modeling (Dai et al, 2002) studies of cat motoneuron behaviour during motor output. Potentially, segregation of ACh effects is due to differential location of cholinergic

inputs in separate compartments of the motoneuron structure. Whereas C-boutons preferentially locate in the soma and proximal dendrites of motoneurons (Witts et al, 2014) where they could be strategically located to modulate AHP properties, our immunohistochemical work suggest that cholinergic inputs on the motoneuronal AIS (Fig. 9) may explain the V_{th} modulation evidenced by our electrophysiological data. Modulation of AIS structure and channel composition has been recently recognized as a novel and powerful mechanism for activity dependent regulation of V_{th} and overall neuronal excitability (see reviews by Grubb et al, 2011and Adachi et al, 2014).

Conclusion

Cholinergic inputs are an important modulatory source for motoneuron firing behaviour. This study indicates that ACh modulates motoneuron V_{th} in a state-dependent fashion, producing more frequently V_{th} hyperpolarization in quiescent networks and V_{th} depolarization in spontaneously active, tonically bursting networks. ACh availability, network activity and other systems influences appear to account for the differential effects of ACh on V_{th}.

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Figure legends

Figure 1. Bath application of acetylcholine can modulate the voltage threshold of lumbar motoneurons.

A: Schematic illustration of a neonatal rat (left) from which the spinal cord was isolated and placed ventral side up in a recording chamber (right). Ventral roots from the second (L2) and fifth (L5) lumbar segments were recorded bilaterally to monitor network activity. Whole-cell patch clamp recordings were obtained from individual antidromically identified lumbar motoneurons. Synaptic blockers were added to the bathing solution (not illustrated) to synaptically isolate the recorded motoneuron. B: Voltage clamp recordings from an L5 motoneuron held at -60.0 mV and subjected to depolarizing voltage steps (+2.0 mV increments) for determination of its voltage threshold (V_{th}). The first fast inward current induced by the smallest depolarizing step is indicated by the arrow. Application of 30 µM acetylcholine (ACh) caused a fast inward current to be elicited at -44.0 mV (red trace, ACh) compared to -40.0 mV in control condition (black trace, left). This -4.0 mV hyperpolarization of V_{th} was reversible (right traces; returned to -40.0 mV after 30 min washout). C: Voltage clamp recording as in B, but for an L2 motoneuron, recorded in a different preparation. ACh caused a reversible +6.0 mV V_{th} depolarization in this cell. V_{th} in control conditions was -30.0 mV (black trace, left) and following ACh application the V_{th} changed to -24.0 mV (blue trace). After 35 min of washout the V_{th} returned to -30.0 mV (black trace, right). The scale bar below panel C applies to both B and C.

Figure 2. Acetylcholine can have different effects on voltage threshold for different lumbar motoneurons.

This graph summarizes the results from 76 experiments in which the effect of bath-applied ACh was assessed on the V_{th} of lumbar motoneurons (as in Figure 1). The effect of ACh on V_{th} is plotted as the relative change in V_{th} following application of ACh and sorted based on the amplitude of change. Blue bars indicate ACh-induced V_{th} depolarization, gray bars indicate no change in V_{th} and red bars indicate ACh-induced V_{th} hyperpolarization. 31 motoneurons showed V_{th} depolarization (+2.0 to +14.0 mV change), 11 showed no change in V_{th} and 34 showed V_{th} hyperpolarization (-2.0 to -24.4 mV change).

Figure 3. The effect of acetylcholine on voltage threshold is independent of its effect on afterhyperpolarization amplitude and repetitive firing properties.

The upper traces show current clamp recordings from an L5 motoneuron made to fire action potentials by application of small (0.2 nA, 250 ms, top traces) and larger (0.5 nA, 1 sec, bottom traces) current steps. Following application of 40 μM ACh (middle trace) the peak AHP amplitude (double headed arrows) was increased from 10.2 to 19.2 mV. This 88% increase was partially blocked by 18 μM of the M_2 antagonist Methoc as the AHP amplitude was reduced to 14.2 mV (right trace) representing a 56% reduction. V_{th} did not change in the three conditions in this motoneuron (-53.0 mV, dotted line), The corresponding changes in the repetitive firing induced by the larger current pulse for this same neuron in the same three conditions are illustrated in the bottom traces. Motoneuron firing was decreased in response to the same amplitude of current injection in the presence of ACh compared with control (mean

firing rate of 4 Hz and 9 Hz, respectively), and it partially recovered after Methoc (mean firing rate of 8 Hz).

Figure 4. The effect of acetylcholine on motoneuron voltage threshold is influenced by the activity state of the network.

A: Raw recordings of the L5 and L2 ventral roots from both left and right sides before (Control, left), and 15 minutes after the addition of 50µM of ACh to the bath (right). Synaptic blockers were present in the solution bathing the spinal cord throughout the recording (not illustrated). In this example the network remained inactive before and after ACh application, as reflected by the lack of spiking or bursting in all roots. An rL5 motoneuron was recorded simultaneously (not shown) under both conditions and its V_{th} values are plotted in B. B shows the V_{th} value prior to (-32.0 mV, left point) and following ACh application (-36.0 mV, right point). This V_{th} hyperpolarization is represented by the red line connecting both points. C: Raw ventral root recordings as in A, but for a different experiment in which spontaneous activity synchronous among 3 roots was present in the control condition, in spite the presence of synaptic blockers in the bath. After ACh application, the occurrence and amplitude of synchronous bursts was slightly reduced. Similar to B, D represents the V_{th} values of an rL5 motoneuron recorded during the activity shown in C. In this motoneuron, ACh induced a +6.3 mV depolarization of V_{th} (blue line). E: Plot showing the percentage of experiments (n = 76) in which ACh induced motoneuron V_{th} hyperpolarization (red bars), V_{th} depolarization (blue bars) or no change in V_{th} (gray bars) and grouped according to the presence or absence of network activity prior to any drug application, as the examples above. ACh tends to induce V_{th}

hyperpolarization in inactive networks and V_{th} depolarization in tonic, synchronously active networks.

Figure 5. Muscarine and edrophonium affect both network output and motoneuron voltage threshold.

A: An example of the raw L2 and L5 (left and right) ventral root activity elicited by 10 μM muscarine after 15 min of bath application. The muscarine induced rhythmic bursting alternated between left and right sides for some periods, but usually lacked consistent L2-L5 or left-right alternation. In addition, the activity often did not include activation of all ventral roots. B: Summary plot of the effect of muscarine on the V_{th} of 10 motoneurons. As in Figure 4, the V_{th} value prior to (Control, left points) and following application of muscarine (right points) for each cell (n = 10) is connected by a line. Red lines represent V_{th} hyperpolarization (6/10), blue lines represent V_{th} depolarization (3/10) and a gray line (n = 1) represents no change in V_{th}. C: The acetylcholinesterase inhibitor EDRO typically induced a single long lasting (0.5-1 min) burst that was synchronous among all ventral roots. D: as in B, this plot summarizes the effect of EDRO on motoneuron V_{th} (n = 8) EDRO produced V_{th} hyperpolarization in 5/8 cells that was associated with the emergence of the burst.

Figure 6. Acetylcholine tends to either not affect or hyperpolarize the voltage threshold of lumbar motoneurons in spinal cords with a reduced network.

A: Schematic illustration of the reduced isolated spinal cord used for assessment of the effects of bath-applied ACh in "reduced network" preparations. Spinal hemi-cords containing only the fourth (L4) and fifth (L5) lumbar segments of one side of the cord were used for network

and individual motoneuron recording. Ventral roots from both lumbar segments were used to monitor network activity while whole-cell patch clamp recordings were obtained from antidromically identified lumbar motoneurons. B: This graph summarizes the results from 16 experiments in which the effect of ACh effect on motoneuron V_{th} was determined. Data is plotted as the relative change in V_{th} following application of ACh and sorted based on the amplitude of change. Blue bars indicate ACh-induced V_{th} depolarization, gray bars indicate no change in V_{th}, and red bars indicate ACh-induced V_{th} hyperpolarization. Most neurons in these reduced network preparations showed either no change in V_{th} after ACh application (7/16), or a V_{th} hyperpolarization (6/16; -4.0 to -8.0 mV change). An additional 3 motoneurons showed V_{th} depolarization (+2.0 to +6.0 mV change).

Figure 7. Antagonists of endogenous muscarinic or nicotinic receptor activity alter motoneuron voltage threshold.

A-B: Plots showing the V_{th} value prior to (left points) and after administration of one cholinergic antagonist (right points) for different lumbar motoneurons. Measurements from the same neuron are connected by a line. Red lines represent V_{th} hyperpolarization, blue lines represent V_{th} depolarization and gray lines represent no change in V_{th}. A: Summary of results for 11 motoneurons in which the muscarinic M_2 receptor antagonist Methoc was directly applied to the bath. Methoc induced V_{th} hyperpolarization in 5/11, V_{th} depolarization in 5/11 and no change in V_{th} in 1/11 motoneurons. B: Summary of results for 9 motoneurons in which the nicotinic antagonist Mecam was tested. Mecam induced V_{th} depolarization in 5/9, V_{th} hyperpolarization in 2/9 and no change in V_{th} in 2/9 motoneurons.

Figure 8. Muscarinic and nicotinic receptor antagonists revert motoneuron voltage threshold changes induced by acetylcholine.

A-D: Plots showing the V_{th} value prior to (left points), after ACh application (middle points) and following the addition of one cholinergic antagonist (right points), from different lumbar motoneurons. Measurements from the same neuron are connected by a line. A: Summary of results for 13 motoneurons in which ACh induced V_{th} hyperpolarization and Methoc was added. Methoc was able to reverse, or partially reverse, the ACh-induced V_{th} hyperpolarization in 10/13 motoneurons (green lines), suggesting M_2 receptors contribute to this effect. B: Summary of results for 11 motoneurons in which ACh induced V_{th} depolarization and Methoc was tested. Again, Methoc reversed, or partially reversed changes in V_{th} induced by ACh in 6/11 neurons (green lines). C-D: Mecam was able to reverse or partially reverse the changes in V_{th} induced by ACh. Mecam reversed ACh-induced V_{th} hyperpolarization in 6/9 neurons (purple lines in C). V_{th} depolarization was reversed in only 1 of 8 neurons (purple line in D), suggesting a weaker involvement of nicotinic receptors in mediating this effect compared with M_2 receptors.

Figure 9. Motoneuron axon initial segments are associated with cholinergic terminals in lumbar segments of the adult rat spinal cord.

Lumbar sections of adult rat spinal cords show vesicular acetylcholine transporter (VAChT, red) immunohistochemical staining in motoneurons located in laminae VIII and IX (A1). The same sections were also stained for Ankyrin G, a marker of axon initial segments (green, A2). The overlay for this section (A3) shows VAChT immunoreactive terminals in the somatodendritic area. Also, a close apposition of VAChT-positive terminals to the axon initial

segment (AIS) of the motoneuron is observed (arrow). B-C: Overlay of 2 additional motoneurons from L2 and L4 lumbar segments, respectively, in which a close association between the AIS and cholinergic terminals was also found (arrows). D: Overlay of an L2 motoneuron in which no obvious association between cholinergic terminals and AIS was present. Scale bar: 25 μm.

Figure 10. Factors influencing state-dependent effects of acetylcholine on the voltage threshold of lumbar motoneurons.

Different states of the network are indicated at the left in panels A-D. ACh concentration (used to refer to both ACh molecule availability and cholinergic receptor activity) is represented by the green background whereas influences from other putative neuromodulatory systems are represented by the yellow background. As indicated by the green and yellow arrows on top, ACh and other systems concentration go from lowest at the left to highest at the right. Neurons connected by black arrows represent a group of experiments in which a cholinergic drug was tested. Drug application is represented as follows: continuous arrows for ACh, dashed arrows for Methoc, dotted arrows for Mecam; thin arrows with open heads for muscarine, and thick arrows with open heads for EDRO. The position of neurons within the horizontal axis indicates the hypothetical ACh concentration affecting the motoneuron before (neuron on the tail side of each arrow) and after (neuron on the head side of each arrow) a cholinergic drug was used. Only the outcome with the highest percentage observed per group of experiments is illustrated; this percentage is shown at the bottom right of the neuron drawings. The effect of ACh concentration on motoneuron V_{th} is coded by the color of the neuron drawings as follows: gray for no change in V_{th}, blue for V_{th} depolarization, and light

and bright red for moderate and strong V_{th} hyperpolarization, respectively. As shown by the coloured bottom arrow, ACh induced V_{th} hyperpolarization at intermediate doses whereas it induced V_{th} depolarization at low and high doses. Refer to text for details.

Figure 1

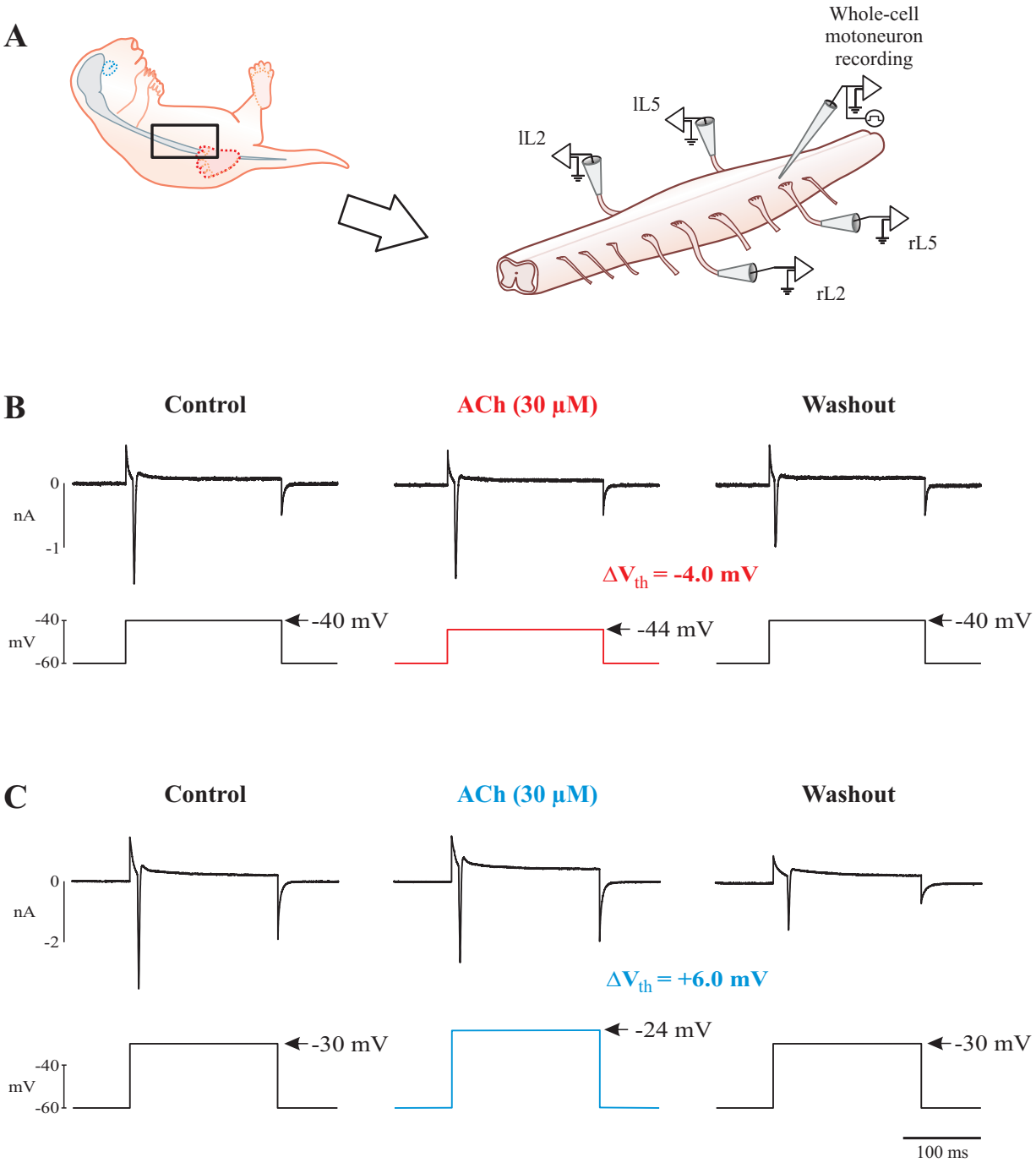

A
Whole-cell
motoneuron
recording
lL5
lL2
rL5
rL2
B
Control
ACh (30 µM)
Washout
0
nA
-1
ΔVth = -4.0 mV
-40
mV
-60
-40 mV
-44 mV
-40 mV
C
Control
ACh (30 µM)
Washout
0
nA
-2
ΔVth = +6.0 mV
-40
mV
-60
-30 mV
-24 mV
-30 mV
100 ms

Figure 2

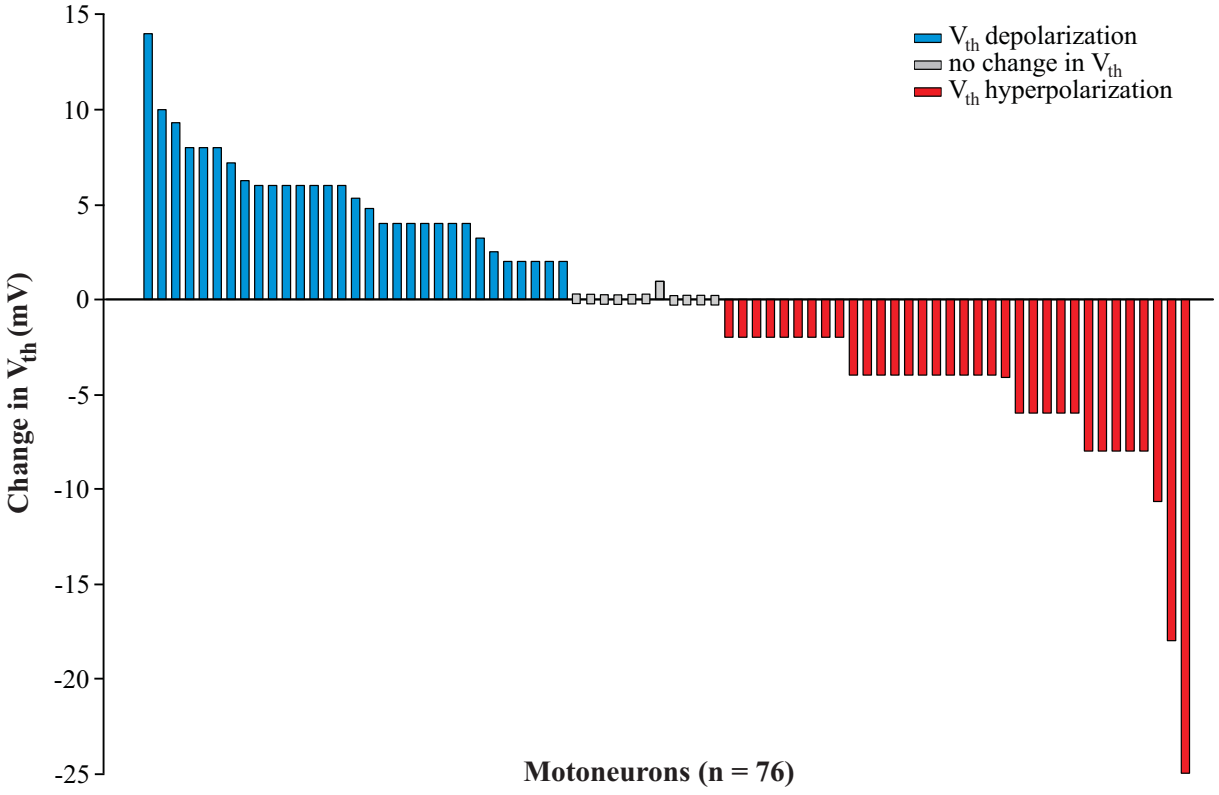

15
10
5
0
-5
-10
-15
-20
-25
Change in V_{th} (mV)
V_{th} depolarization
no change in V_{th}
V_{th} hyperpolarization
Motoneurons (n = 76)

Figure 3

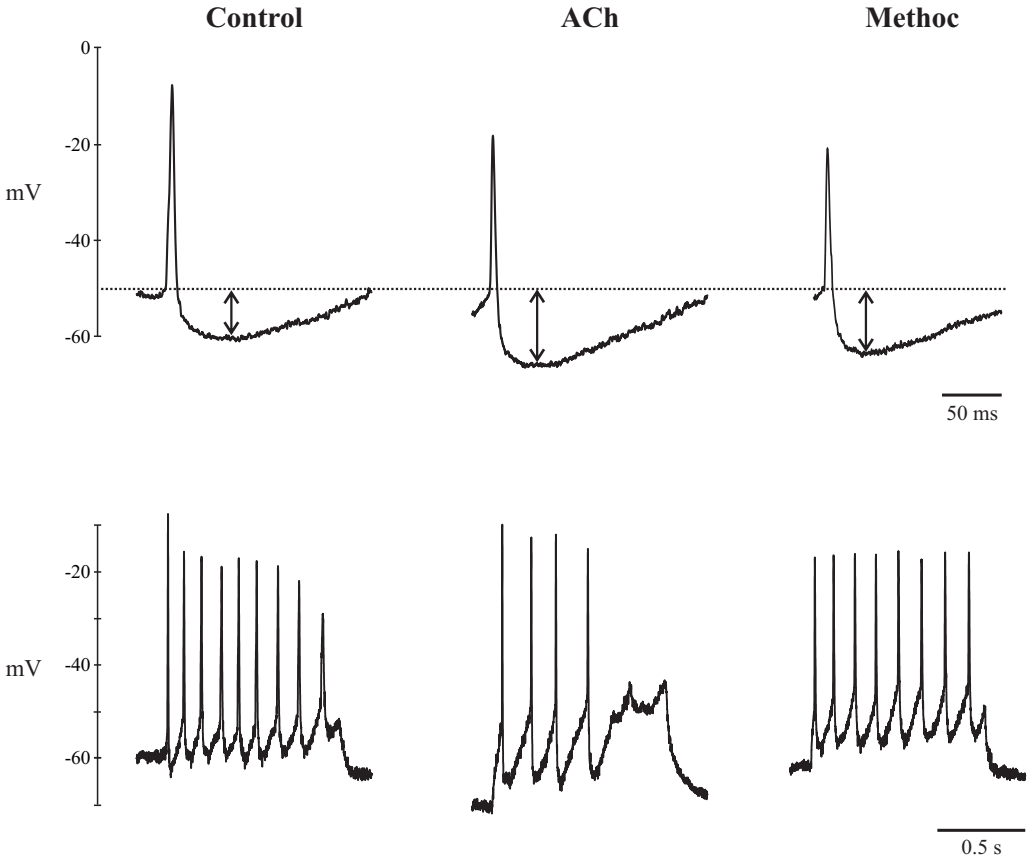


Figure 4

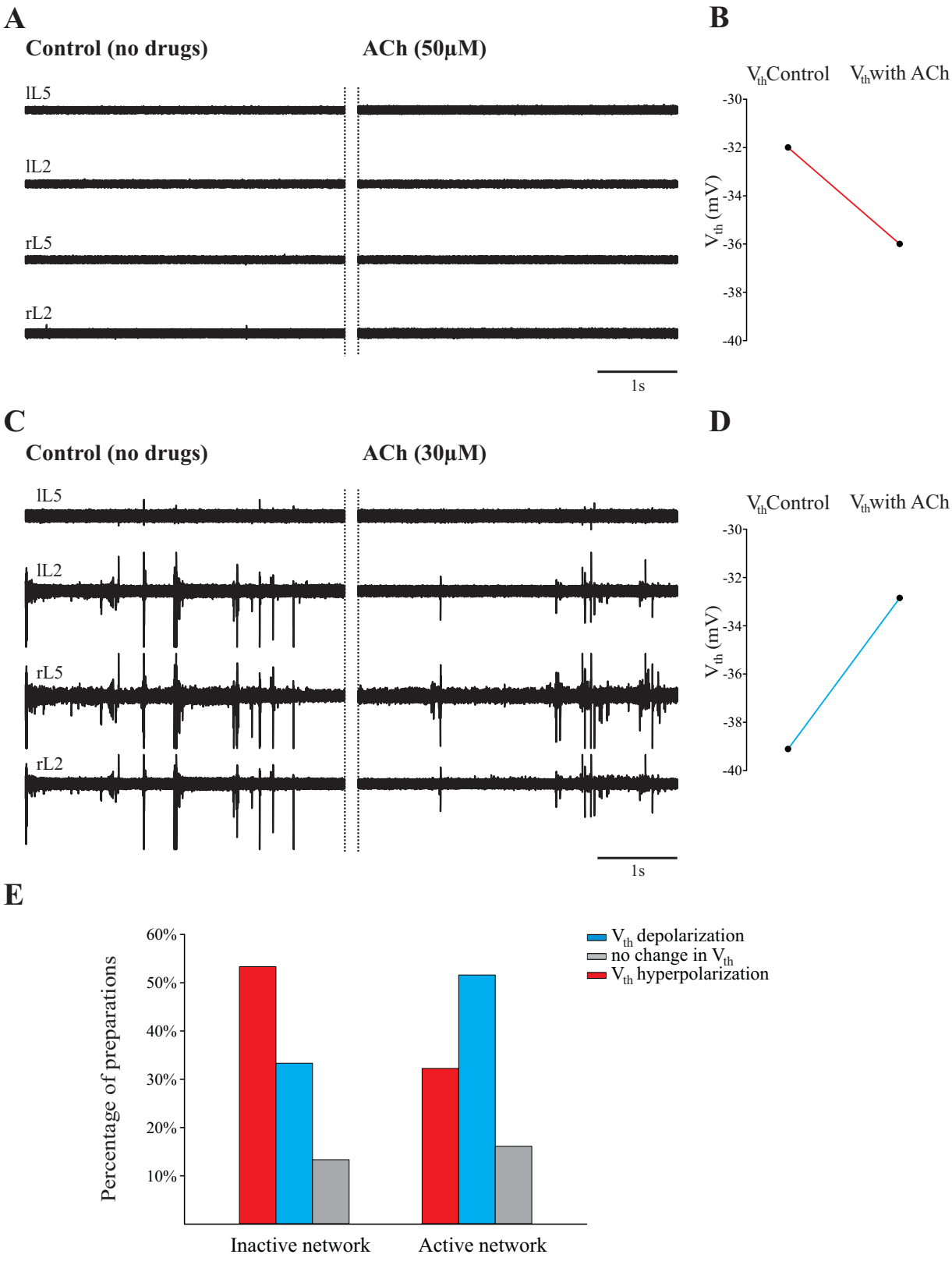

A
Control (no drugs)
ACh (50µM)
lL5
lL2
rL5
rL2
1s
B
V_{th}Control
$V_{th}$with ACh
-30
-32
-34
-36
-38
-40
V_{th} (mV)
C
Control (no drugs)
ACh (30µM)
lL5
lL2
rL5
rL2
1s
D
V_{th}Control
$V_{th}$with ACh
-30
-32
-34
-36
-38
-40
V_{th} (mV)
E
60%
50%
40%
30%
20%
10%
Percentage of preparations
V_{th} depolarization
no change in V_{th}
V_{th} hyperpolarization
Inactive network
Active network

Figure 5

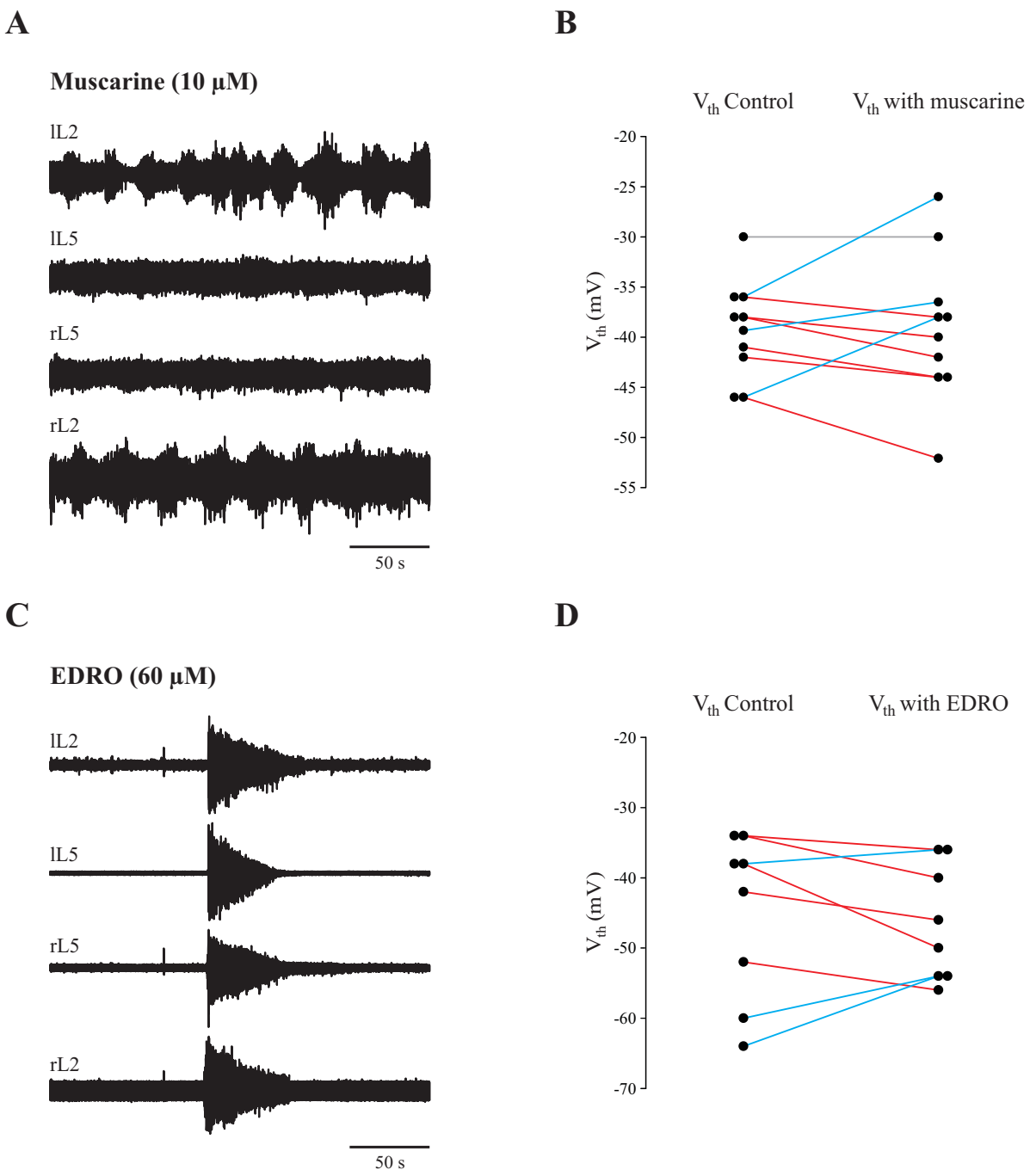
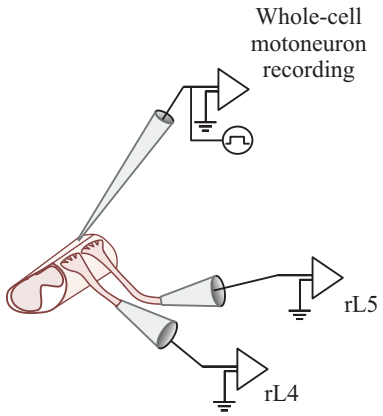

A
Muscarine (10 µM)
lL2
lL5
rL5
rL2
50 s
B
V_th Control
V_th with muscarine
V_th (mV)
-20
-25
-30
-35
-40
-45
-50
-55
C
EDRO (60 µM)
lL2
lL5
rL5
rL2
50 s
D
V_th Control
V_th with EDRO
V_th (mV)
-20
-30
-40
-50
-60
-70

Figure 6

A


Whole-cell
motoneuron
recording
rL5
rL4

B

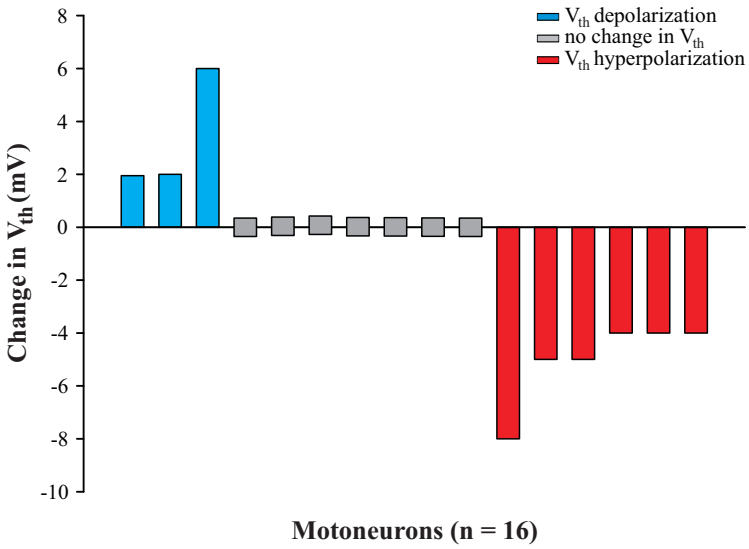

V_{th} depolarization
no change in V_{th}
V_{th} hyperpolarization
Change in V_{th} (mV)
8
6
4
2
0
-2
-4
-6
-8
-10
Motoneurons (n = 16)

Figure 7

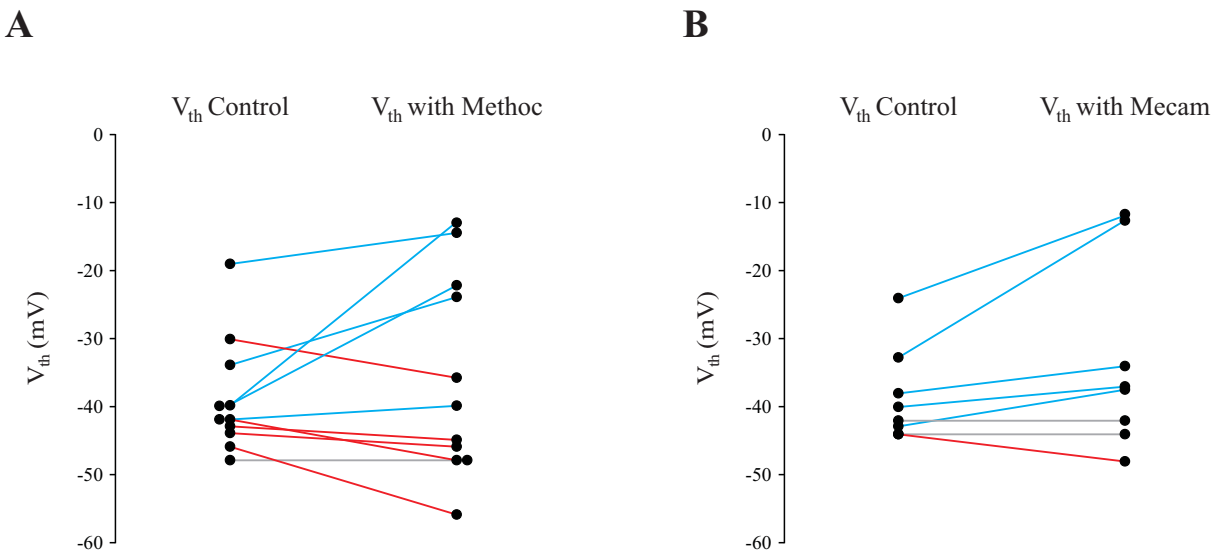

A
B
V_{th} Control
V_{th} with Methoc
V_{th} with Mecam
V_{th} (mV)
0
-10
-20
-30
-40
-50
-60

Figure 8

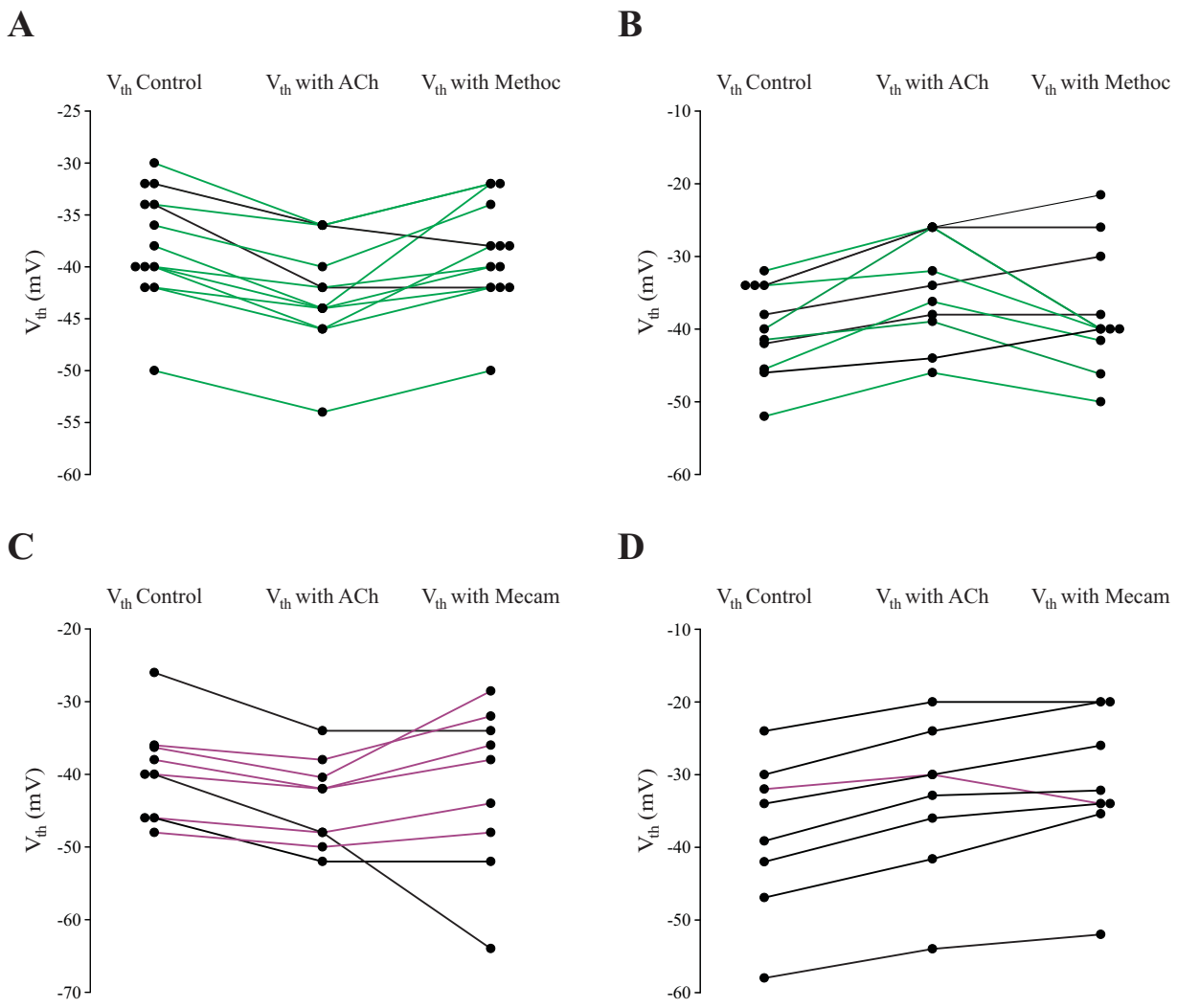

A
B
C
D
V_{th} Control
V_{th} with ACh
V_{th} with Methoc
V_{th} with Mecam
V_{th} (mV)
-25
-30
-35
-40
-45
-50
-55
-60
-10
-20
-70

Figure 9

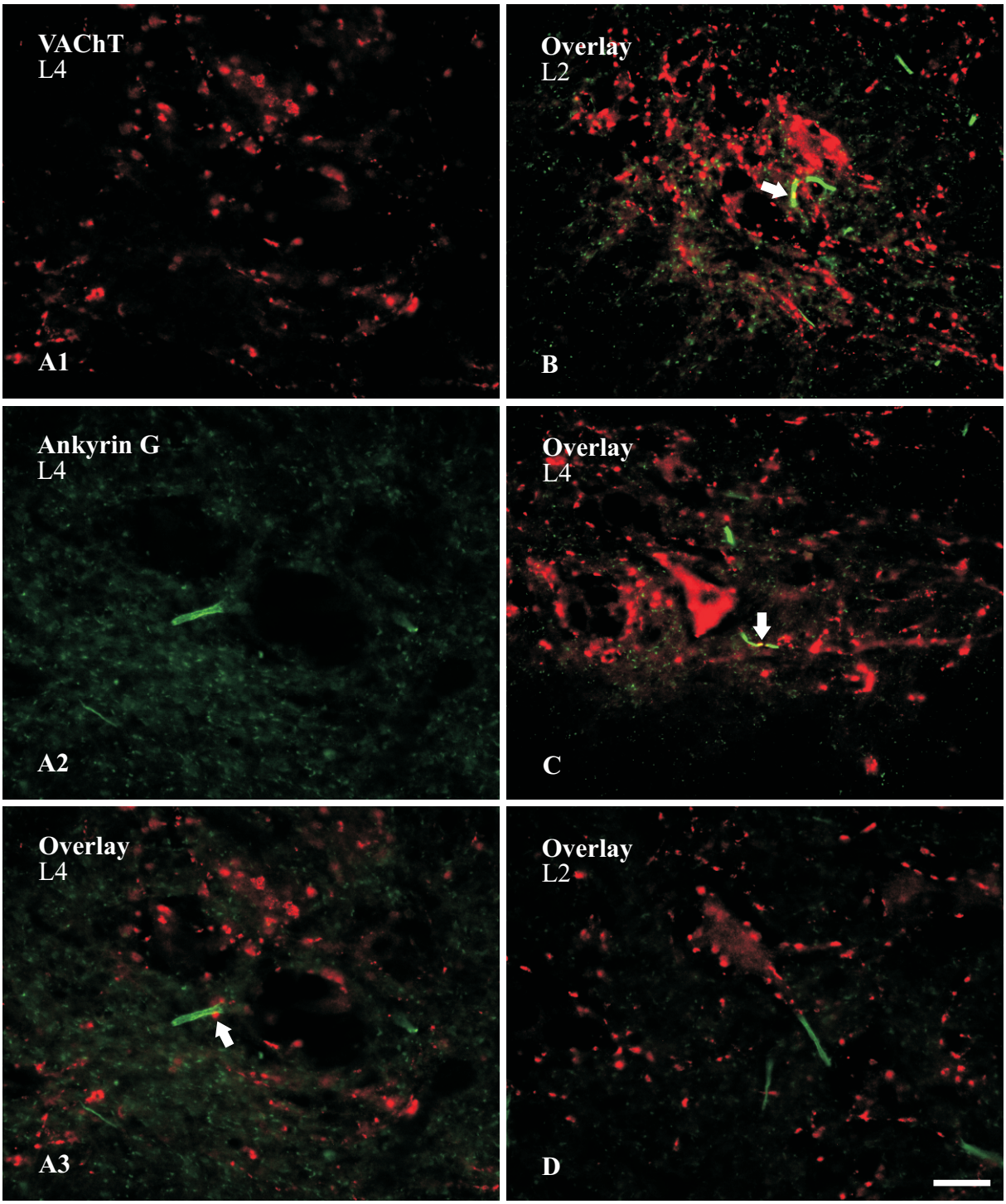
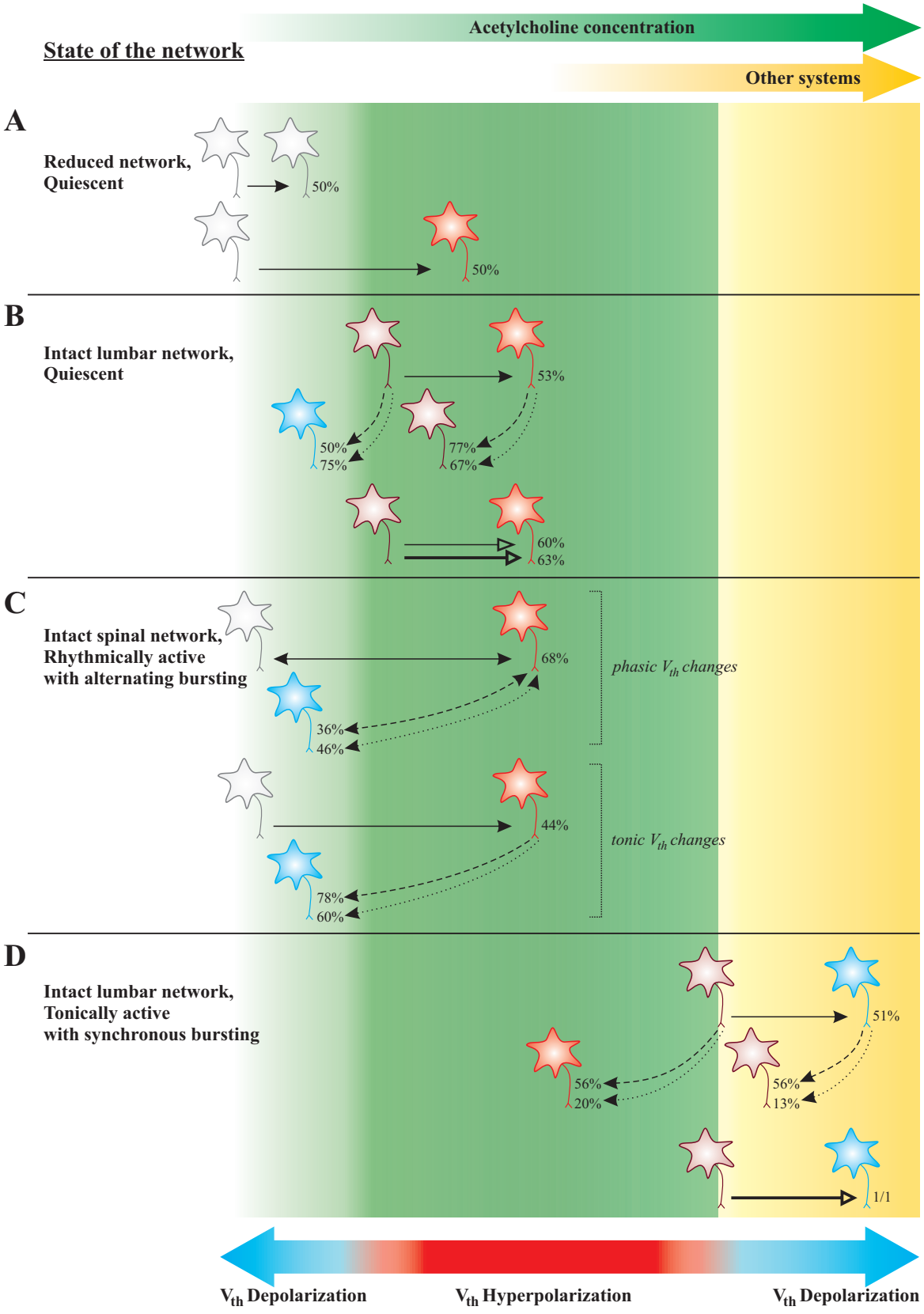

VAChT
L4
A1
Overlay
L2
B
Ankyrin G
L4
A2
Overlay
L4
C
Overlay
L4
A3
Overlay
L2
D

Figure 10


Acetylcholine concentration
Other systems
State of the network
A
Reduced network,
Quiescent
50%
50%
B
Intact lumbar network,
Quiescent
53%
50%
75%
77%
67%
60%
63%
C
Intact spinal network,
Rhythmically active
with alternating bursting
68%
36%
46%
phasic V_{th} changes
44%
78%
60%
tonic V_{th} changes
D
Intact lumbar network,
Tonically active
with synchronous bursting
51%
56%
20%
56%
13%
1/1
V_{th} Depolarization
V_{th} Hyperpolarization
V_{th} Depolarization

Chapter III.

PAPER 2:

State-dependent modulation of motoneuron voltage threshold by intraspinal cholinergic mechanisms

Edna E. Vasquez-Dominguez & Brent Fedirchuk

Abstract

Previous work has demonstrated an intraspinally mediated enhancement of motoneuron excitability during rhythmic motor output in decerebrate cats (Power et al, 2010). The present report extends these findings in an *in vitro* preparation and provides the first evidence that cholinergic mechanisms intrinsic to the spinal cord importantly contribute to this modulation by altering the voltage threshold for action potential initiation (V_{th}). Using a similar approach to that used to elicit fictive scratch in cats, cervical segment stimulation with an agonist of spinal itch receptors (GRP_{18-27}) was used in the isolated spinal cord from neonatal rats to elicit a rhythmic motor pattern in the absence of descending input or chemical stimulation of the lumbar cord. Blind patch whole-cell recordings from antidromically identified lumbar motoneurons showed a reversible hyperpolarization of V_{th} (mean -5.1 mV) during ipsilateral rhythmic network output induced by GRP_{18-27}. The prevalence of V_{th} hyperpolarization was high, occurring in all motoneurons examined, including flexor and extensor pools. When examining individual motoneurons with repeated trials during an experiment, the incidence of observed V_{th} hyperpolarization was also high. Overall it was observed in 68% of all trials. The remaining 32% of trials represented cases where V_{th} hyperpolarization might have been predicted by ongoing motor activity, however V_{th} was not observed. A depolarization of V_{th} (mean +2.7 mV) was observed in association to contralateral rhythmic network output in 80% of motoneurons. M_2 muscarinic or nicotinic receptor blockade in lumbar cord segments, although not completely blocking all state-dependent V_{th} changes during motor output, significantly reduced the incidence of these changes (from 63% to 36% and 75% to 46%, respectively). This suggests that, although both muscarinic and nicotinic receptor subtypes mediate state-dependent changes in motoneuron

V_{th}, their action is not essential for these changes to occur. We conclude that a cholinergic mechanism is active and contributes to motoneuron modulation during spinal motor output. The cholinergic system then may constitute one of the intraspinal neuromodulatory systems that regulates motoneuron excitability associated with the production of rhythmic motor behaviour.

Introduction

Motoneurons are the "last order" or "output" neurons specialized in transforming neural activity into muscular contraction to produce movement (Sherrington, 1906). Motoneuron activity integrates inputs from supraspinal and afferent sources as well as inputs from spinal networks capable of generating organized patterns of motor activity called central pattern generators (CPGs). Far from passively relaying motor commands, the excitability of motoneurons is adjusted by neuromodulators to suit motor tasks. For example, the voltage threshold for action potential initiation (V_{th}) of lumbar motoneurons is hyperpolarized prior to and throughout fictive locomotion (Krawitz et al, 2001) and scratch (Power et al, 2010) in the decerebrated cat preparation. Facilitation of motoneuron recruitment and firing through V_{th} hyperpolarization also occurs during brainstem-evoked locomotor activity in the *in vitro* neonatal rat spinal cord preparation (Gilmore & Fedirchuk, 2004) and the *in vivo* adult rat (MacDonell et al, 2015). Gilmore & Fedirchuk (2004) implicated descending serotoninergic mechanisms in mediating state-dependent V_{th} modulation because the serotoninergic antagonist ketanserin or cooling of the cervical cord blocked this effect. The observation that exogenous application of serotonin (5-HT) or noradrenaline (NA) induced V_{th}

hyperpolarization in ventral horn neurons (Fedirchuk & Dai, 2004) further supported the involvement of descending monoaminergic actions. Recently, however, we demonstrated that the V_{th} hyperpolarization associated with fictive scratch in decerebrate cats persists following acute transection of the cervical cord (Power et al, 2010). This study provided the first evidence that, in addition to descending projections, an intrinsic spinal system has the capability of quick and reversible regulation of motoneuron excitability during movements produced by spinal CPGs. The neuromodulatory identity of this intraspinal system has yet to be determined.

Due to its widespread presence within the spinal cord (Barber et al, 1984; Phelps et al, 1984; Borges & Iversen, 1986) and its involvement in locomotor activity (see Jordan and Schmidt, 2002; Miles & Sillar, 2011; and Witts et al, 2014 for review), the propriospinal cholinergic circuitry constitutes a strong candidate for mediating spinal modulation of motoneuron output. Cholinergic spinal neurons active during locomotion include neurons in laminae VII (partition cells) and X (central canal cluster cells) (Carr et al, 1994, 1995; Huang et al, 2000; Tillakaratne et al, 2014). In addition, cholinergic influences modulate the electrical properties of neurons involved in the control of locomotion (Dai et al, 2009a; Dai & Jordan, 2010). Further, although the spinal and brainstem cholinergic systems appear not to be required for the induction of locomotion (Jordan et al, 2014), endogenous activation of the propriospinal cholinergic network can produce locomotor-like activity in the neonatal rat spinal cord preparation (Smith & Feldman, 1987; Smith et al, 1988; Cowley & Schmidt, 1994; Kiehn et al, 1996; Anglister et al, 2008; Jordan et al, 2014). At the motoneuron level, studies in mammalian lumbar motoneurons have shown an excitatory effect of cholinergic agonists albeit via unspecific mechanisms (Zieglgansberger and Reiter, 1974; Zieglgansberger and

Bayerl, 1976; Evans, 1978; Jiang & Dun, 1986; Blake et al, 1987; Newberry & Connolly, 1989; Kurihara et al, 1993; Ogier et al, 2004). More recent studies showed that cholinergic C-terminals on motoneurons, which originate from a subgroup of Pitx2+ $V0_C$ spinal neurons active during locomotion, enhance lumbar motoneuron excitability by reducing their afterhyperpolarization (AHP) (Miles et al, 2007; Zagoraiou et al, 2009). Additionally, we have recently shown that cholinergic agonists induce motoneuron V_{th} hyperpolarization during specific states of the motor network, and through mechanisms that are likely independent from AHP modulation (Vasquez-Dominguez & Fedirchuk, in preparation). Miles et al (2007) demonstrated that enhancement of endogenous cholinergic actions on motoneurons increased their output during chemically-induced locomotor-like activity; conversely, blockade of muscarinic receptor function resulted in a decrease in locomotor-related burst amplitude. However, because locomotor activity in this preparation was induced by lumbar application of a mix of 5-HT, glutamatergic and dopaminergic agonists, it was not defined whether the ability of the cholinergic system to modulate motoneuron excitability is independent of monoaminergic action. To date, no study has been performed to determine whether intraspinal cholinergic mechanisms mediate changes in motoneuron excitability during motor output independently of descending monoaminergic influences, and specifically through actions on the recruitment V_{th}.

The *in vitro* neonatal rat spinal cord is a suitable model for the pharmacological characterization of the modulatory pathways involved in the regulation of lumbar motoneuron excitability. This preparation generates a variety of motor patterns including locomotor-like activity (Smith and Feldman, 1987; Kudo and Yamada, 1987; see Clarac et al, 2004 for review), myoclonic-like activity (Simon, 1995; Cowley et al, 2002; Pflieger et al, 2002), tail-

flipping (Lev-Tov et al, 2000; Lev-Tov & Delvolve, 2000) and other less regular motor patterns produced by peptides (Suzue et al, 1981; Pearson et al, 2003; Barriere et al, 2005). Induction of these motor outputs, however, requires either drug application to the spinal cord including lumbar segments, or electrical stimulation of the brainstem or dorsal roots. These approaches complicate the assessment of the pharmacological properties of lumbar motoneuron modulation as well as the independence of these mechanisms from descending actions. Therefore, the first aim of the present study was to establish whether the *in vitro* neonatal rat spinal cord is capable of producing a scratch-like rhythmic motor output using a similar approach to that used in the *in vivo* acute spinal cat (as in Domer & Feldberg, 1960; Deliagina et al, 1975; Power et al, 2010). Our second aim was to determine whether state-dependent modulation of motoneuron V_{th} occurs during spinally-generated motor activity as reported in Power et al (2010) and, finally, our third aim was to establish whether intraspinal cholinergic inputs mediate effects in V_{th} independently from descending influences. Portions of this work have been presented in preliminary form (Vasquez-Dominguez et al, 2010; Vasquez-Dominguez & Fedirchuk, 2012).

Methods

All surgical and experimental protocols were conducted in compliance with the guidelines for the ethical treatment of animals issued by the Canadian Council for Animal Care and approved by the University of Manitoba Animal Ethics Committee. A schematic illustration of the experimental preparation is shown in Figure 1.

Surgical procedures

Neonatal Sprague-Dawley rats (postnatal day 0-5; n = 58) were anesthetized with isoflourane in a chamber. After a rapid precollicular/postmammillary mechanical decerebration, rats were moved to a dissecting dish filled with 4°C dissecting artificial cerebral spinal fluid (dACSF) superfused with 95% O_2-5% CO_2 under pressure. After the thorax and abdomen were eviscerated, a dorsal and ventral laminectomy exposed the spinal cord and brainstem. The dura matter covering the medulla and spinal cord was then removed and all spinal roots were cut near the dorsal root ganglia. A transverse transection was performed separating the medulla from the first cervical (C1) segment of the spinal cord and the complete spinal cord was then transferred to a Sylgard-bottomed recording chamber and pinned ventral side up with 0.1 mm insect pins.

Bath partitions

Barriers of acetate film sealed at the recording chamber and petroleum jelly at the spinal cord contact edges were used to divide the bath into cervical and thoracolumbar partitions. Barrier leakage into the thoracolumbar partition was prevented by building a first barrier at the C2-C3 segment division and a second barrier at the C5-C6 segment division. As an additional precaution, a lower fluid level (and therefore lesser hydrostatic pressure) was maintained on the C1-C2 bath partition. Absence of barrier leakage was confirmed at the beginning and end of experiments by draining one side of the barrier and using the microscope to look for fluid seepage across the barrier. Once bath partitions were mounted, the dACSF was replaced with recording artificial cerebral spinal fluid (rACSF) and allowed to slowly warm at room temperature (22-24°C).

Electrical cervical stimulation

C1 dorsal column stimulation was performed with a rACSF filled glass electrode, with a tip diameter of 200-300 µm placed in contact with the ventral surface of the C1 spinal segment. Monophasic rectangular current pulses (5 ms, 0.4 – 1.0 mA, 100 – 200 Hz) were delivered using bipolar stimulation.

Ventral root recording and analysis

Ventral root recordings from the second and fifth lumbar ventral roots on both the left and right sides (lL2, lL5, rL2, rL5) were obtained using suction electrodes (A-M systems, #573000). Ventral root records were band-pass filtered (30-3000 Hz), digitized at 5 kHz and captured using a PC-based data acquisition and analysis platform with special purpose software (developed by the Spinal Cord Research Centre, University of Manitoba). Raw recordings from the ventral roots containing sustained GRP_{18-27}-induced rhythmic activity were analyzed in order to determine the relationships among bursts of activity recorded from pairs of ventral roots. Circular statistics were used to establish these relationships, as described in Kriellaars et al (1994), Kjaerulff & Kiehn (1996), Cowley et al (2005), Liu & Jordan (2005). In brief, the phase values of bursts of activity from L2 and L5 homolateral ventral roots are used to evaluate flexor/extensor coordination. It is widely accepted that L2 and L5 ventral root activity mostly corresponds to activity in flexor and extensor motoneuron pools, respectively (Cowley and Schmidt, 1994; Kjaerulff and Kiehn, 1996). The phase values of bursts of activity from bilateral ventral roots are used to examine right/left coordination. Phase values are displayed graphically as data points on a polar plot and the mean phase value is displayed as a vector. The length of the vector, *r*, ranges from 0 to 1 and represents the

concentration of data around that vector. Dispersed data points indicate no phase relationship, and concentrated data points indicate coupling of discharge in the two ventral roots under comparison. Significant concentration between data points was determined using Raleigh's circular statistical test, so that of *r* was greater than the critical Raleigh's value (cR) for a given P value, the ventral root activity was considered phase-related.

Intracellular recordings

Motoneurons in the L2 and L5 lumbar segments were targeted for single-cell recordings using borosilicate glass microelectrodes pulled with a Narishige PP-83 two-stage puller and filled with patch electrode solution. The filled electrodes had resistances ranging from 2.7 to 4.5 MΩ. After making a small pial incision at L2 or L5 level, the microelectrode was introduced into the ventral horn from the ventral surface of the spinal cord. A whole-cell single cell recording arrangement was obtained using the 'blind patch' technique (Blanton et al, 1989). In all experiments, the ventral root corresponding to the spinal segment containing the neuron being recorded was electrically stimulated (180-560 μA) in an attempt to activate the neuron antidromically. Only data from antidromically activated motoneurons are reported. A Multiclamp 700A patch-clamp amplifier, Digidata 1200 A/D converter, and pClamp 9.0 software (all from Axon Instruments) were used for data acquisition. Series resistance was monitored (usually <40 MΩ) to ensure that it did not change significantly during the recording period. Neither series resistance nor tip potentials were compensated. Data were sampled at 10 kHz.

Solutions and chemicals

Extracellular solutions: The dACSF contained (in mM): NaCl (25), sucrose (88), KCl (1.9), NaH_2PO_4 (1.2), $MgSO_4$ (10), $NaHCO_3$ (26), kynurenic acid (1.5), D-glucose (25), and $CaCl_2$ (1.0). The rACSF contained (in mM): NaCl (125), KCl (2.5), $NaHCO_3$ (26), NaH_2PO_4 (1.25), D-glucose (25), $MgCl_2$ (1), and $CaCl_2$ (2.5). The pH of these solutions was adjusted to 7.3 with KOH. Osmolarity was adjusted to 305 mOsm by adding sucrose to the solution.

Electrode solution: The patch electrode solution contained (in mM): K-gluconate (120), NaCl (5), HEPES (10), EGTA (5), $MgCl_2$ (2), CaCl (1), Mg-ATP (5), and GTP-Na_2 (0.5). KOH was used to adjust the pH to 7.3 and osmolarity was adjusted to 305 mOsm.

Drugs: Drug application was by means of addition to the perfusate and included the following: bicuculline (10 – 300 µM), strychnine (20 – 80 µM), tubocurarine (10 – 200 µM), N-methyl-D,L-aspartate (NMDA, 10 – 100 µM), gastrin-releasing peptide agonist 18-27 (GRP_{18-27}, 0.1 nM – 1 µM), methoctramine (20 – 80 µM), and mecamylamine (20 – 100 µM). All drugs, except the peptide GRP_{18-27}, were obtained from Sigma and were initially dissolved in distilled water and stored frozen at -20°C as 10 mM stock solutions. GRP_{18-27} was obtained from Chemicon and stored as 1 mM stock solution. To obtain the selected final bath concentration of a drug, stock solution was directly added in fractionated amounts with the use of a micropipette at the periphery of the stationary chamber. All concentrations specified in this report, including ranges, refer to the final bath concentration.

Measurement of V_{th}

Both, current-clamp and voltage-clamp records were included in the sample. In current-clamp, the V_{th} for eliciting an action potential can be directly measured from the

voltage record as the membrane potential at the point of maximal change of voltage (set at ≥10 mV/s in the present study) at the onset of an action potential evoked by either a pulse or ramp of injected depolarizing current. V_{th} was defined as the value of membrane potential of the data point that was ≥1 mV more depolarized than the preceding point because a 10 kHz digitization rate was used throughout the present study (Power et al, 2010). In effect, this method is analogous to choosing the point at which the slope of the first derivative of membrane potential exceeds 10 V/s. In voltage-clamp mode, the set of events underlying an action potential are observed as a fast inward current. In this configuration, an initial holding potential of -60.0 mV was used to approximate the resting membrane potential of the neuron, and 200-ms depolarizing pulses were successively delivered in +2.0 mV increments at a repetition rate of 2 Hz. Fast inward currents which would have mediated action potentials in current-clamp mode were evident on the recorded current trace and the potential of the smallest depolarizing step capable of inducing a fast inward current was defined as the V_{th} (Fedirchuk and Dai, 2004). A hyperpolarization of V_{th} manifests as a smaller depolarizing voltage step (i.e. more negative membrane potential) being able to induce a fast inward current, whereas V_{th} depolarization is the opposite (i.e. a larger voltage step is required, and V_{th} is a less negative membrane potential). It has been previously demonstrated that V_{th} measured in voltage clamp are tightly correlated with those measured in current clamp (Dai et al, 2009b). Prior to drug application, repeated trials of the protocol for measuring V_{th} ensured that the control V_{th} remained stable. Following the start of rhythmic activity, V_{th} was measured during the periods of ventral root quiescence as well as the periods of ventral root activity. This measurement continued after a cholinergic antagonist was applied into the thoracolumbar bath partition. Consequently, in this study each neuron served as its own

control. To ensure that a neuron's response had not been altered by a preceding drug exposure, only one neuron was recorded for each spinal cord preparation.

Statistical differences in V_{th} before and after a pharmacological manipulation were assessed with a paired *t*-test. When multiple conditions were compared (e.g., following consecutive drug applications or during different motor activity states), one-way repeated measures analysis of variance (one-way RM ANOVA) tests were performed. For all tests, $P < 0.05$ was considered a significant difference.

Results

A novel model for the assessment of spinally-mediated modulation of motoneuron excitability during motor output

In decerebrate cats, fictive scratching is elicited after application of small pieces of cotton soaked in curare (0.1 – 0.3%), strychnine (0.5%) or bicuculline (1-2 mg/ml) on the dorsal root entry zone of the uppermost cervical segments of the spinal cord (C1 and C2) and following manual stimulation of the pinna (Domer & Feldberg, 1960; Deliagina et al, 1975, 1981; Degtyarenko et al, 1998; Power et al, 2010). To simulate this approach using the neonatal rat preparation, isolated spinal cords were placed in a partition dish separating the first two cervical segments of the spinal cord. This arrangement allowed selective exposure of C1-C2 segments to different neurochemicals while the bathing solution of the lumbar spinal cord remained unchanged (recording ACSF only). While in the cat preparation bouts of fictive scratching are elicited after manual stimulation of the skin around the ear and neck, we

performed cervical afferent stimulation through a suction electrode placed on the C1 dorsal columns. The motor output was recorded using suction electrodes attached to the ventral roots from the second (L2) and fifth (L5) lumbar segments on both sides of the spinal cord. Figure 1 illustrates the experimental arrangement used for this project. In addition to curare, strychnine and bicuculline, other drugs were tested in their ability to elicit rhythmic motor activity when bath-applied to the cervical cord either alone or in combination with other drugs or electrical stimulation of the cervical dorsal columns.

Cervical drug application facilitates a fast frequency motor output in response to C1-dorsal column stimulation

Prior to any drug application, the motor response to C1-dorsal column (DC) stimulation was assessed in all experiments (n = 35). A representative example of the raw ventral root output resulting from C1-DC stimulation in the absence of drugs is illustrated in Figure 2A. Although the tonic activity in all lumbar ventral roots was slightly increased after the electrical stimulation, this effect outlasted the stimulation only for a few seconds. In some preparations (22/31), DC stimulation alone evoked brief periods of high frequency rhythmic discharge (>4 Hz) along with tonic activity (not shown). However, this rhythmic activity was not present in all ventral roots and faded quickly after the stimulation ceased. The effects of the addition of different neurochemicals to the bath partition surrounding cervical segments of the cord on the evoked and spontaneous lumbar ventral root output are summarized in Table 1. Application of bicuculline (10-200 μM; n = 4) to C1-C2 spinal segments evoked long-lasting bilateral episodes of fast frequency (4-12 Hz) bursts in response to DC stimulation, as the representative example in Figure 2B. A similar response was obtained for all 4

experiments where strychnine (10-80 μM) was initially applied alone to the cervical bath and later followed by addition of bicuculline (not illustrated). The duration and robustness of the responses to strychnine and/or bicuculline usually took several minutes to develop and the frequency of the rhythmic bursts decayed from 12 to 4 Hz within seconds after cessation of the electrical stimulus in all cases. The fast frequency response occurred simultaneously in both left and right ventral roots and could co-exist with slower (<0.5 Hz) rhythmic bursts (3/8), as the example in Figure 2B. Fast frequency bursts could be out of phase, sometimes having clear ipsilateral L2/L5 alternation; however, periods of synchronous activity within the course of each experiment were often observed (inset in Figure 2B). In all 8 preparations, increasing doses of strychnine or bicuculline seemed not to have a significant effect in the response robustness and/or rhythmic pattern (Table 1). In 4 preparations, we assessed the effects of a non-specific increase of spinal excitability by administration of potassium chloride (KCl, 2.5 mM). Increases in cervical KCl concentration evoked a similar effect as bicuculline and/or strychnine in all preparations examined, as the example illustrated in Figure 2C. The response to KCl and DC stimulation was not altered by addition of other neuroactive compounds (including NMDA, curare, strychnine and/or bicuculline) (Table 1). Therefore, the enhancement of the excitability of spinal C1-C2 segments induced by GABAergic or glycinergic blockers, or via unspecified pathways, resulted in the facilitation of a fast frequency rhythmic discharge evoked by electrical stimulation of the C1 dorsal columns. This activity, however, lacked consistent left/right or flexor/extensor alternation. Curare (30-50 μM) elicited similar ventral root activity patterns following DC stimulation as those described above, either when applied alone (n = 4) or in combination with bicuculline or other compounds (n = 6). However, it was observed that at higher doses (>50 μM) curare

additionally induced spontaneous activity (4/5). This activity consisted of high frequency bursts (~7 Hz) occurring either ipsilaterally or simultaneously in all ventral roots, as observed in the representative example depicted in Figure 3A. The curare-induced motor pattern in our neonatal rat preparation lacked clear left/right and flexor/extensor alternation (inset in Figure 3A) that are characteristic of the well-coordinated feline fictive scratch pattern.

Activation of spinal itch receptors in the cervical cord elicits spontaneous rhythmic motor activity

Gastrin-releasing peptide receptors (GRPRs), present in lamina I of the dorsal horn, have recently been shown to act as specific mediators of the itch sensation (distinguishable from the pain sensation) in the mouse spinal cord (Sun & Chen, 2007; Sun et al, 2009). Here we also tested the GRPR ligand, GRP_{18-27}, as a specific stimulus to elicit scratch-like behaviour in our neonatal rat preparation. Similar to the effects of curare, after brief exposure of C1-C2 spinal segments to GRP_{18-27}, spontaneous rhythmic bursts were observed in all preparations (n =14). A representative example of the GRP_{18-27}-induced motor pattern in the absence of cervical DC stimulation is illustrated in Figure 3B. Unlike curare however, GRP_{18-27}-induced bursts occurred in either the left or right ventral roots, not bilaterally at the same time (Figures 3B and 4A). Figure 4A shows another example of the activity pattern induced by cervical application of GRP_{18-27} at a long time scale. Analysis of the phase relation between individual bouts of activity (or "slow bursts") in the left and right L5 ventral roots confirmed the observation that, although the onset of bursting in one side of the cord is relatively variable, it is restricted to quiescence in the contralateral side (Figure 4B, left plot). On the other hand, polar plot analysis of the right L2 and L5 ventral roots revealed that the onset of

slow bursts for ipsilateral roots are strongly synchronized (Figure 4B, right plot), indicating co-activation of flexor/extensor motor pools. Each individual bout of GRP_{18-27}-induced activity or "slow burst" consisted of fast frequency rhythmic discharges ("fast bursts") of 4-6 Hz that were distinguishable when observed at a fast time scale, as shown in Figure 5A. As for the slow bursts, there was some synchrony between the flexor and extensor activity (L2 and L5 ventral root output, respectively) of ipsilateral fast frequency discharges (Figure 5B). The pattern consisting of synchronous flexor/extensor and alternating left/right ventral root slow and fast bursts was observed in all preparations (14/14) in which GRP_{18-27} was applied to the cervical cord.

Although a wide range of GRP_{18-27} doses was tested (0.1 nM – 1 μM), it was observed that 200-500 nM were optimal for producing spontaneous rhythmic bursting that lasted for approximately 30 min. Once GRP_{18-27} concentration reached values within the 200-500 nM range, it elicited sustained rhythmic motor activity in most preparations (10/14). Lower doses of GRP_{18-27} (<200 nM) did not alter the ventral root output or occasionally induced a few ventral root bursts that were short in duration, small in amplitude and occurred in only one or two ventral roots (3/6). Higher doses of GRP_{18-27} (>1 μM) first increased the occurrence of rhythmic episodes followed by increases in episode duration (5/5). After long exposure to the peptide, however, slow intermittent bursting appeared in co-existence with the fast-frequency episodes and later this developed into tonic ventral root discharge. This tonic motor output appeared in most preparations in which GRP_{18-27} was present in the cervical compartment for more than ~40 min (11/14), independently of whether moderate (200-500 nM) or high (>1 μM) doses were used. Although the motor pattern elicited by GRP_{18-27} may not strictly correspond to scratch as described in the cat (see Rossignol, 1996 for review), it allows the

study of intraspinal modulation of motoneuron V_{th} during motor activity. GRP_{18-27}-induced activity is a spinally-generated rhythmic motor output produced independently of supraspinal descending influences and of chemical or electrical stimulation of the lumbar spinal cord. These features render this novel preparation suitable for the assessment of whether the circuitry intrinsic to the spinal cord is capable of modulating motoneuron excitability associated to rhythmic motor activity, while it also allows the pharmacological manipulation of the lumbar motoneuron environment for the assessment of the neuromodulator systems involved in this control.

Intraspinally-mediated state-dependent modulation of motoneuron V_{th} occurs in the neonatal rat preparation

Using the same preparation described above, GRP_{18-27} was applied to the C1-C2 bath partition while whole-cell patch clamp recordings from lumbar motoneurons were obtained and their V_{th} assessed prior to and during rhythmic activity. Motoneuron recordings were performed in either the voltage clamp (14/27) or current clamp (13/27) configuration. Prior to GRP_{18-27} application to the cervical bath compartment, repeated trials were obtained to ensure that the control V_{th} remained stable. The absolute value of V_{th} determined using the voltage- and current-clamp protocols ranged from -20.2 to -52.0 mV (mean = -36.0 ± 8.0 mV).

Motoneuron V_{th} hyperpolarization occurs during ipsilateral bouts of rhythmic network output

A representative example of a motoneuron recorded prior to and during a bout of ipsilateral GRP_{18-27}-induced motor activity is shown in Figure 6A. This motoneuron was

located in the left side of the L5 segment (lL5 motoneuron) and network bursting occurred first in the ventral roots ipsilateral to the neuron (lL2 and lL5 ventral roots). Two identical series of depolarizing steps of +2.0 mV increments from the initial holding potential of -60.0 mV were delivered in order to determine the smallest depolarizing step able to induce a fast inward current. Steps to more depolarized holding potentials invariably elicited a fast inward current. As shown in Figure 6B, a step to -38.0 mV was required to elicit the first inward current and was the V_{th} for this neuron during the quiescent period of ventral root output (black trace). During the bout of ipsilateral ventral root activity, the first fast inward current was elicited at -44.0 mV (red trace). This difference represents a -6.0 mV hyperpolarization of the V_{th}. This effect was reversible because the V_{th} returned to -38.0 mV after the ipsilateral activity burst had ended (not illustrated). Motoneuron V_{th} hyperpolarization associated to ipsilateral rhythmic bursting was also evident by spontaneous action potential firing in lumbar motoneurons (Figure 7) and was observed independently of whether motoneurons were recorded in voltage- or current-clamp configuration. Further, during the GRP_{18-27}-induced activity, rhythmic membrane fluctuations could be observed in association to ipsilateral network output in some (see for example Figure 7A) but not all motoneurons. V_{th} hyperpolarization occurred in all motoneurons independently of the presence of these rhythmic membrane fluctuations and whether the motoneurons were recruited into firing or not.

State-dependent V_{th} hyperpolarization was observed in all motoneurons studied (27/27). This change in V_{th} was significant (paired *t*-test; $P \leq 0.001$) and ranged from -2.0 to -19.2 mV (mean = -5.2 ± 4.0 mV). The V_{th} averages from each motoneuron under 3 different conditions are illustrated in Figure 8A: control (open circles), control after GRP_{18-27}

application (GRP-quiescence; black circles) and ipsilateral rhythmic activity (GRP-active; red circles). It is evident from this plot that although the mean Control V_{th} could either depolarize or hyperpolarize after $GRP_{18\text{-}27}$ was added to the cervical compartment, the mean V_{th} from ipsilateral bursting activity ($\dot{x}V_{th}$ GRP-active) was hyperpolarized with respect to the mean V_{th} from periods of ventral root quiescence ($\dot{x}V_{th}$ GRP-quiescence) in most motoneurons (24/27; 89%).

The prevalence of V_{th} hyperpolarization was high, occurring in all motoneurons examined, including flexor and extensor pools. However, when examining individual motoneurons with repeated trials during an experiment, it was common to observe that most ipsilateral bursts were accompanied with motoneuron V_{th} hyperpolarization, while occasionally some bursts were not associated with a change in V_{th}. Figure 16B shows the incidence of observed V_{th} hyperpolarization for each motoneuron, where for each bar the remaining percentage represents the number of cases where V_{th} hyperpolarization might have been predicted by ongoing motor activity, however a change in V_{th} was not observed. The incidence (i.e. reproducibility) of state-dependent V_{th} hyperpolarization varied from experiment to experiment, and ranged from 56 to 100% (mean 68%).

Motoneuron V_{th} depolarization occurs during contralateral bouts of rhythmic network output

Power et al (2010) reported that, contrary to the motoneuron V_{th} hyperpolarization that occurs during ipsilateral fictive scratch in cat preparations, V_{th} depolarization is observed during fictive weight support that occurs during contralateral scratch (see their Figure 6). This demonstrated that modulation of motoneuron V_{th} is specific to the motor state. Interestingly, in the neonatal rat preparation, we also observed that motoneuron V_{th} could depolarize during

contralateral ventral root activity induced by GRP_{18-27}. Figure 9 shows a representative example of a lumbar motoneuron recorded prior, during and after a GRP_{18-27}-induced contralateral ventral root burst. The lL5 motoneuron in this example showed a reversible +2.0 mV depolarization of V_{th} from -38.0 to -36.0 mV associated to contralateral motor output, denoted by the requirement of a larger step to elicit a fast inward current during contralateral network activity (blue trace) compared to network quiescence present prior to and after this activity.

V_{th} depolarization associated with contralateral bursting was observed in 16/20 motoneurons (80%). The magnitude of the depolarization ranged from +2.0 to +6.9 mV (mean = +2.8 ± 1.7 mV). In contrast to the state-dependent V_{th} hyperpolarization reported above, the V_{th} depolarization was a less consistent observation in all experiments. For the motoneurons in which V_{th} depolarization was observed, we repeated the assessment and calculated the incidence at which the observation was detectable. The V_{th} depolarization incidence ranged from 25 to 83% (mean = 44%).

Intraspinal cholinergic mechanisms contribute to the state-dependent modulation of motoneuron V_{th}

The V_{th} changes observed in association with GRP_{18-27}-induced rhythmic bursting in this study support the idea that mechanisms intrinsic to the spinal cord are capable of quick and reversible modulation of motoneuron excitability associated with rhythmic motor output. We tested the hypothesis that intrinsic modulation of motoneuron V_{th} is at least partly

mediated by a spinal cholinergic system by attempting to block state-dependent V_{th} changes with cholinergic antagonists.

For this series of experiments, we used the same experimental setting described above in which GRP_{18-27} is applied to the cervical bath partition while either voltage (9/14) or current (5/14) clamp recordings from individual lumbar motoneurons are obtained. After observing a few examples of state-dependent motoneuron V_{th} hyperpolarization within 5-10 min following cervical GRP_{18-27} application, a cholinergic antagonist was then applied in increasing doses to the dish partition bathing the lumbar segments of the spinal cord. Either the M_2 muscarinic antagonist, methoctramine (20-80 μM) or the nicotinic antagonist, mecamylamine (20-100 μM) was tested at a time. Motoneuron V_{th} was again assessed to evaluate whether V_{th} changes associated to rhythmic network output were prevented by the presence of the antagonist.

Cholinergic antagonists block motoneuron V_{th} hyperpolarization associated with rhythmic motor output

The ability of methoctramine to block state-dependent motoneuron V_{th} hyperpolarization was assessed in 9 experiments. Methoctramine could either completely or partially block V_{th} hyperpolarization in several episodes of GRP_{18-27}-induced ipsilateral activity; however, this observation was not consistent for all V_{th} changes observed in the course of a single experiment. A representative example is illustrated in Figure 10. As in previous examples, top traces in panels A-C show the ventral root activity induced by GRP_{18-27} while the simultaneous motoneuron recording is shown in the bottom trace. The V_{th} of this motoneuron prior to any drug application was -50.0 mV from a -60.0 mV holding potential (not shown). Soon after GRP_{18-27} application into the cervical cord (and in absence of drugs in

the lumbar compartment), rhythmic network activity started and motoneuron V_{th} hyperpolarization (from -50.0 to -52.0 mV) was observed in association to ipsilateral bursting (Figure 18A). Methoctramine (20 µM) was then applied to the lumbar bathing solution, while GRP_{1827}-induced rhythmic output was still ongoing. Measurement of V_{th} from the same motoneuron under this new condition showed no difference in the values assessed both prior and during ipsilateral bursting (Figure 18B), suggesting that the antagonist blocked the network-induced V_{th} hyperpolarization. Later in the experiment, however, another case of V_{th} hyperpolarization associated to ipsilateral ventral root activity was found (Figure 18C). This state-dependent V_{th} change occurred in spite methocramine concentration was higher (40 µM) and the V_{th} associated to ventral root quiescence was lower than those of previous moments of the experiment. As in this example, most experiments showed some degree of state-dependent motoneuron V_{th} hyperpolarization remnant after methoctramine application. Similar to methoctramine, lumbar bath application of mecamylamine resulted in blockage of some but not all V_{th} hyperpolarizations associated to GRP_{18-27}-induced rhythmic bursting (not shown). These observations suggest that both M_2 muscarinic and nicotinic receptors are involved in mediating state-dependent V_{th} changes but that their action is not required for these changes to occur. Neither methoctramine nor mecamylamine seemed to affect the GRP_{18-27} motor pattern or the amplitude of bursting.

Cholinergic antagonists block most but not all state-dependent motoneuron V_{th} changes

To assess whether cholinergic antagonists produced a significant reduction in the number of state-dependent V_{th} hyperpolarization, data from each experiment was first plotted in the form of V_{th} averages from five different conditions (Figure 11): 1) control, prior to

application of any drugs (Control no drugs, open circles); 2) ventral root quiescence after GRP_{18-27} was applied to the cervical bath and rhythmic motor output had started (GRP-quiescence, black circles); 3) ipsilateral ventral root rhythmic activity induced by GRP_{18-27} (GRP-active, red circles); 4) ventral root quiescence after an antagonist was applied to the lumbar bath while GRP_{18-27}-induced rhythmic activity was ongoing (GRP+Methoc quiescence and GRP+Mecam quiescence for methoctramine and mecamylamine, respectively; gray circles); and 5) ipsilateral ventral root rhythmic activity in the presence of the antagonist (GRP+Methoc active (green circles) and GRP+Mecam active (blue circles) for methoctramine and mecamylamine, respectively). The plot in Figure 11A contains the V_{th} averages from experiments in which methoctramine was used, whereas experiments using mecamylamine are summarized in Figure 11B.

Although statistical comparison of the average V_{th} measured during the different conditions did not show any clear effect of the antagonists on the V_{th} hyperpolarization associated to ipsilateral activity, some trends emerged. The average control V_{th} measured during the periods of ventral root quiescence after antagonist application (GRP+Methoc/Mecam quiescence) was slightly depolarized with respect to its homonymous average prior to the antagonist (GRP-quiescence) in most experiments (10/14; 71%). This suggests that spinal network activation by GRP_{18-27} may include activation of cholinergic receptors in the motoneuron that set its excitability in a different level despite the absence of motor output. More importantly, it is observed that whereas the GRP-active average is moderately hyperpolarized with respect to the GRP-quiescence average prior to antagonist application, this difference is not observed in the homonymous averages once the antagonists were present (GRP+Methoc/Mecam quiescence *vs* GRP+Methoc/Mecam active). Although

this is observed in only 6/10 experiments, it is an indication of the ability of cholinergic antagonists to block the V_{th} hyperpolarization associated to rhythmic motor output.

As mentioned in the previous section, the state-dependent V_{th} hyperpolarization observed in our preparation was not entirely consistent for all cases within the course of an experiment. This can therefore confound the assessment of the ability of an antagonist to block the state-dependent V_{th} changes. For this reason, we compared the state-dependent V_{th} hyperpolarization incidence rates before and after antagonist application for each experiment, as shown in Figure 12. It is observed that whereas the incidence of state-dependent V_{th} hyperpolarization before methoctramine was between 56 and 90%, these rates dropped to less than 50% in all experiments (9/9; Figure 12A) after methoctramine administration. Statistical comparison of the overall incidence before (mean = 63%) and after (mean = 36%) methoctramine resulted in a significant reduction of these rates after the antagonist (paired *t*-test; $P \leq 0.001$). The incidence of V_{th} hyperpolarization in the presence of methoctramine ranged from 22 to 50%. The average percent reduction was 44% and ranged from 12 to 61%. The incidence rates of state-dependent V_{th} hyperpolarization for experiments in which mecamylamine was tested are shown in Figure 12B. Similar to methoctramine, mecamylamine produced a reduction in the proportion of V_{th} hyperpolarization associated with rhythmic bursting in all experiments (5/5). The mean incidence prior to (mean = 75%) and after (mean = 46%) mecamylamine was also statistically reduced (paired *t*-test; $P \leq 0.05$). The incidence of V_{th} hyperpolarization before mecamylamine ranged from 56 to 100%, whereas in the presence of the antagonist this rate dropped to between 0 to 90%. The mean percent reduction was 46% (range = 5 – 100%).

It was reported earlier in this study that, in addition to V_{th} hyperpolarization associated with ipsilateral bursting, motoneuron V_{th} depolarization associated with contralateral bursting was observed in several experiments. From the experiments in which cholinergic antagonists were tested in their ability to block state-dependent motoneuron V_{th} changes, V_{th} depolarization was only observed in 3 experiments with methoctramine and 3 experiments with mecamylamine. Methoctramine showed a tendency to reduce the mean V_{th} depolarization incidence rate from 44% to 29%. This difference, however, was not statistically significant (paired *t*-test; $P = 0.43$). Similarly, mecamylamine showed a non-statistically significant (paired *t*-test; $P = 0.28$) trend to decrease the mean V_{th} depolarization incidence from 53% to 29%. Overall, this data suggests that the intraspinal cholinergic system is involved in the modulation of motoneuron V_{th} associated to the production of rhythmic motor output. Although both M_2 muscarinic and nicotinic receptors mediate these state-dependent changes in V_{th}, and because their blockade does not completely block these effects, other mechanisms appear to also contribute to this modulation.

Discussion

This study is the first to show that intraspinal cholinergic inputs to lumbar motoneurons mediate V_{th} hyperpolarization during rhythmic motor behaviour independently of influences from descending modulatory systems. We used an *in vitro* neonatal rat spinal cord preparation to induce rhythmic motor output independently of electrical or chemical activation of supraspinal or lumbar neuronal structures to first demonstrate that systems intrinsic to the spinal cord exert state-dependent modulation of motoneuron V_{th}. Then we

showed that most changes in V_{th} associated to rhythmic network output were prevented or reduced by muscarinic or nicotinic acetylcholine receptor blockade, implicating these receptors in intraspinal modulation of motoneuron excitability.

The present investigation was undertaken to extend our previous findings (Power et al, 2010) that motoneuron V_{th} is hyperpolarized by intraspinal mechanisms during fictive scratch in acutely spinal transected cats. To mimic the cutaneous stimulation used to trigger the scratch reflex in cats, we used an agonist of receptors mediating itch transmission in the spinal cord, GRP_{18-27} (Sun & Chen, 2007; Sun et al, 2009). Although this peptide did not generate a motor pattern that strictly corresponded to scratch as described in the cat (see Rossignol, 1996 for review) or as observed in unrestricted neonatal rat pups (Vasquez-Dominguez & Fedirchuk, unpublished observations), the GRP_{18-27}-elicited rhythmic motor output allowed us to study how motoneuron excitability is regulated as a result of spinal rhythm generating network operation.

State-dependent modulation of motoneuron V_{th}

The motoneuron V_{th} hyperpolarization observed during rhythmic activity in the present study (mean -5.1 mV) is similar to that observed during brainstem-induced locomotion in neonatal rats (mean -6.0 mV; Gilmore & Fedirchuk, 2004), adult rats (mean -3.7 and -5.8 mV for motoneurons with and without locomotor drive potentials (LDPs), respectively; MacDonell et al, 2015), and adult cats (mean -8.0 mV; Krawitz et al, 2001). Also, the V_{th} hyperpolarization occurring during cervically-evoked ipsilateral rhythmic activity reported here resembles that described by Power et al (2010) during ipsilateral fictive scratch in spinal intact (mean -5.4 mV) and spinal transected (mean -7.1 mV) decerebrate cats. As in all these

studies, the V_{th} hyperpolarization reported here occurred for the first action potential, recovered quickly after cessation rhythmic output and included a wide range of change (-2.0 to -19.2 mV).

During locomotion and scratch, motoneurons demonstrate depolarizing and hyperpolarizing fluctuations in their membrane potential (LDPs; scratch drive potentials, SDPs, respectively) and V_{th} becomes hyperpolarized during both the depolarizing and hyperpolarizing phases of these potentials (Krawitz et al, 2001; Power et al, 2010). During the GRP_{18-27}-induced activity, V_{th} hyperpolarization occurred in all motoneurons independently of the presence of rhythmic membrane fluctuations or whether the motoneurons were silent during network activity. These observations are consistent with Krawitz et al (2001) and MacDonell et al (2015) findings that the V_{th} hyperpolarization during locomotion is not correlated to amplitude of the LDP observable in motoneurons. Our present data provides further support for the idea that enhancement of motoneuron excitability is produced when the motor network is activated, independently of whether that individual motoneuron is actively involved in the production of the motor output or not.

The prevalence of V_{th} hyperpolarization in the present study (27/27 motoneurons = 100%) was as high as reported in the aforementioned studies (cat locomotion: 100%; adult rat locomotion: 100%, neonatal rat locomotion: 91%; spinal intact cat scratch: 79%; spinal cat scratch: 92%). However, we observed that the incidence of this change for all rhythmic activity bouts elicited during the course of each experiment was lower (mean 68%). The remaining 32% represented trials where V_{th} hyperpolarization might have been predicted by ongoing motor activity but this change in V_{th} was not observed. Although currently unclear, this inconsistency in state-dependent V_{th} hyperpolarization occurrence in our preparation is

potentially due to a limited ability of spinal preparations to modulate V_{th} during motor behaviour, or differences in the modulatory pathways activated according to the type of motor output (compare prevalence from cat locomotion *vs* scratch, for example). Alternatively, the lack of consistency may be explained by developmental limitations of the neonatal rat preparation (compare prevalence from adult *vs* neonatal rat locomotion, for example). In spite these dissimilarities, however, V_{th} hyperpolarization seems to constitute a mechanism readily used by the motor system to facilitate motoneuron recruitment that is preserved across different species, developmental stages and a variety of rhythmic motor behaviours.

Power et al (2010) showed that whereas V_{th} hyperpolarization occurs during rhythmic activation of motoneurons during ipsilateral fictive scratch, a depolarization of V_{th} is observed during tonic activation of extensor motoneurons during fictive weight support that occurs during contralateral scratch. This change in V_{th} (mean +3.7 mV) was reversible and occurred in 86% of motoneurons studied. Further supporting a task-specific modulation of motoneuron excitability, MacDonell et al (2015) argued that the V_{th} hyperpolarization observed during the tonic network output preceding the start of brainstem-induced locomotion in adult rats appear to be the result of mechanisms favouring locomotion *vs* stance. In the present report, V_{th} depolarization occurred in association with contralateral bouts of GRP_{18-27}-induced rhythmic activity (mean +2.7 mV) in 80% of motoneurons. This change, however, occurred without the presence of tonic motor output in the ipsilateral ventral roots that might be analogous to fictive weight support (Fig. 9), which might be due to postural deficits of rats during the first week after birth (Clarac et al, 1998, 2004; Vinay et al, 2000). Nevertheless, our data suggests that, as in adults, mechanisms exist at early developmental stages to restrict increases in excitability

to motoneurons innervating a limb engaged in a particular motor task, and suggests that this modulation is site and state-specific.

Intraspinal regulation of motoneuron excitability

In addition to V_{th}, other properties are regulated to facilitate motoneuron firing during rhythmic motor output. For example, during fictive locomotion and/or scratch lumbar motoneurons exhibit a reduction in rheobase current (Krawitz et al, 2001), a decreased afterhyperpolarization (AHP) amplitude (Brownstone et al, 1992; Schmidt, 1994; Power et al, 2010), a suppression of spike frequency adaptation (Brownstone et al, 2011; MacDonell et al, 2015) and the emergence of persistent inward currents (PICs) facilitating nonlinear integrative properties (Brownstone et al, 1994; Power et al, 2010). Until recently, the modulation of motoneuron excitability was thought to be dependent upon descending monoaminergic inputs. Evidence for this came from demonstrations that the voltage-dependent amplification of synaptic input mediated by PICs is eliminated following acute thoracic transection and recovered via administration of monoaminergic precursors in decerebrate cats (Hounsgaard et al, 1988; Conway et al, 1988). Further, acute administration of 5-HT or NA agonists in this preparation facilitates emergence of PICs (Heckman et al, 2003). Similarly, Gilmore & Fedirchuk (2004) showed that cooling of the cervical cord or application of the 5-HT_2 antagonist to the spinal cord eliminated the V_{th} hyperpolarization during brainstem-induced locomotion in neonatal rats. Also, monoamines induce motoneuron V_{th} hyperpolarization in this preparation (Fedirchuk & Dai, 2004; Han et al, 2007; Tartas et al, 2010). It was thus a surprising finding that changes in motoneuron properties including voltage-dependent amplification of SDPs, AHP amplitude reduction and V_{th} hyperpolarization persist during

fictive scratch in cats acutely spinally transected at cervical segments (Power et al, 2010). It was evident, however, that intraspinal modulatory systems activated in parallel or as part of the scratch CPG exerted state-dependent modulation of motoneuron excitability. Results in the present report are consistent with Power et al (2010) observations that acute enhancement of motoneuron excitability during rhythmic motor activation occurs in preparations devoid of supraspinal input. Analogous to fictive scratch in the spinal cat, we used stimulation of cervical segments of isolated neonatal rat spinal cords without a brainstem to elicit a rhythmic motor output triggered and generated exclusively by spinal networks, and we found that reversible V_{th} hyperpolarization occurs in association to the production of this rhythm. In contrast to scratch (Sherrington, 1910) and the GRP_{18-27}-induced rhythm, the initiation of locomotion in experimental preparations requires the activation several supraspinal centers (see Jordan et al, 2008 for review) and thus it is possible that distinct modulatory systems are predominantly activated during these two motor behaviours. Nevertheless, as in Power et al (2010), the present study highlights that, in addition to descending monoaminergic influences, an intraspinal modulatory system is capable of quick, reversible and task-specific modulation of motoneuron excitability.

Intraspinal cholinergic modulation of motoneuron excitability

Although anatomical studies have suggested that cholinergic inputs to motoneurons constitute an important modulatory source, only recently have the physiological mechanisms underlying this modulation begun to be described (Frank, 2009; Witts et al, 2014). Miles et al (2007) demonstrated that activation of muscarinic M_2 receptors, which are clustered at postsynaptic sites formed by cholinergic terminals called C-boutons (Muennich & Fyffe,

2004; Hellstrom et al, 2003; Wilson et al, 2004), increase lumbar motoneuron excitability by reducing a SK-type Ca^{2+}-dependent K^+ current which decreases AHP amplitude. These authors further showed that enhancement of endogenous cholinergic actions increased the burst amplitude of mixed 5-HT, NMDA and dopamine-induced locomotor-like activity, whereas blockade of muscarinic receptors decreased the burst amplitude (Miles et al, 2007). Although this group subsequently demonstrated the intraspinal origin of cholinergic neurons generating C-boutons (restricted to a subpopulation of Pitx2+ $V0_C$ partition neurons located lateral to the central canal) and showed that inactivation of this input reduced the enhancement of motoneuron firing during a motor task (Zagoraiou et al, 2009), it remained to be determined whether cholinergic modulation of motoneuron excitability was exerted independently of descending monoaminergic influences. In the present study, we elicited a rhythmic motor output in the absence of supraspinal structures and monoaminergic agonist application to the spinal cord, to exclusively activate intraspinal systems. The motoneuron V_{th} hyperpolarization occurring during rhythmic network activity was significantly reduced following cholinergic receptor blockade. To our knowledge, this represents the first demonstration of the ability of intraspinal ACh sources to modulate motoneuron excitability during motor output.

Supporting a role for ACh in V_{th} modulation, we have shown in a separate study that bath application of ACh to lumbar networks in the absence of motor output can induce motoneuron V_{th} hyperpolarization (Vasquez-Dominguez & Fedirchuk, in preparation). The degree of V_{th} change was similar to that reported here (mean -5.5 mV) and thus is consistent with that induced by CPG activation. Moreover, our preliminary immunohistochemical work in that study revealed the presence of cholinergic terminals in close proximity to the axon initial segment (AIS) of some adult rat lumbar motoneurons, strongly suggesting that

cholinergic modulation is exerted on the motoneuronal AIS compartment which could mediate the influence on V_{th} that we observe in our electrophysiological recordings (Vasquez-Dominguez & Fedirchuk, in preparation). Interestingly, ACh application in such study was also able to induce V_{th} depolarization in motoneurons when the spinal network was exhibiting spontaneous tonic activity, suggesting that the state of the network activity influences cholinergic actions on motoneuron V_{th}.

Although severely reduced, the incidence of state-dependent changes in V_{th} in the present report was not completely abolished by M_2 muscarinic or nicotinic receptor antagonists (Fig. 12). This indicates that, although both muscarinic and nicotinic receptor subtypes are involved in mediating V_{th} hyperpolarization associated with rhythmic motor activity, they likely do not mediate the complete modulatory effect on their own. This also probably explains why, in contrast to Miles et al (2007), we did not detect changes in ventral root burst amplitude. It is thus possible that other intraspinal systems, such as glutamatergic inputs acting at metabotropic receptors which are known to affect V_{th} (Iwagaki & Miles, 2011), may also contribute to facilitating motoneuron recruitment in the absence of descending modulatory inputs. Future investigations are needed to determine the interaction between distinct modulatory systems, including intraspinal and descending sources, in relation to the modulation of V_{th} and other electrical properties influencing motoneuron excitability. Further, whether different modulatory systems are selectively or redundantly activated during diverse motor tasks needs to be explored.

Conclusion

The present study extends previous observations of motoneuron V_{th} modulation associated with the production of rhythmic network motor output mediated by intraspinal mechanisms. Further, we have provided the first evidence that the spinal cholinergic system importantly contributes to this modulation. A "hyper-cholinergic" state developed a few days following spinal cord injury has been suggested to excessively enhance motoneuron output preventing the recovery of locomotion in mammals (Jordan et al, 2014), underscoring the need for investigation of the mechanisms involved in cholinergic modulation of V_{th} and other motoneuron electrical properties.

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Figure legends

Figure 1. Experimental paradigm used for the generation of a spinally-induced rhythmic motor output in the *in vitro* neonatal rat spinal cord preparation. For elicitation of fictive scratch in the cat preparation, curare or strychnine is topically applied to the first (C1) and/or second (C2) cervical segments of the spinal cord while the pinna is manually stimulated (not illustrated). Using this approach, spinal cords isolated from neonatal rats were placed in a partition dish isolating the bathing solutions between the C1-C2 cervical segments (coloured red) from the rest of the spinal cord (coloured blue), for selective neurochemical application. In some experiments a suction electrode was used for electrical stimulation of the dorsal columns of the uppermost cervical segments. Motor output was recorded using suction electrodes bilaterally placed on the ventral roots from the second (L2) and fifth (L5) lumbar segments.

Figure 2. Increasing the excitability of the upper cervical spinal segments facilitates a fast frequency rhythmic lumbar motor output in response to electrical stimulation at the first cervical segment.

A: Raw ventral root output of the second (L2) and fifth (L5) lumbar (left and right) segments produced in response to C1-dorsal column electrical stimulation (DC stim bar at the top; stimulus artifacts are truncated for illustration purposes) in the absence of drugs. B: DC-evoked rhythmic activity in the presence of 70 µM bicuculline in C1-C2 segments of the spinal cord. C: Facilitation of the DC-evoked rhythmic activity by a slight increase in the extracellular potassium concentration (KCl, 2.5 mM) in C1-C2 spinal segments. Except for insets, scale is shown in B.

Figure 3. Cervical application of curare or GRP_{18-27} elicits spontaneous fast frequency rhythmic activity of the lumbar motor network.

A: Example of the raw ventral root output produced by application of curare (100 µM) to C1-C2 segments of the spinal cord. This activity did not require electrical dorsal column stimulation and it consisted of bursting activity in either one or both sides of the cord. No clear left/right or L2/L5 alternation was consistently observed (inset). B: As in A, example of the spontaneous activity elicited by the peptide GRP_{18-27} (520 nM), which activates receptors signaling itch in the spinal cord (Sun & Chen, 2007; Sun et al, 2009). Bursts occurred simultaneously in the L2 and L5 ventral roots (inset) and alternated between left and right sides. Except for insets, scale is shown in A.

Figure 4. Bouts of GRP_{18-27}-induced rhythmic activity occur bilaterally, but occur on only one side at a time.

A: Raw ventral root activity induced by cervically applied GRP_{18-27} (250 nM), without electrical stimulation. B: Polar plots depicting the phase relation for the bouts of activity (slow bursts) in left and right L5 ventral roots (left plot) and the right L2 and right L5 ventral roots (right plot). The 0 position on the polar plot corresponds to the onset of activity in the right L5 (rL5, left plot) and L2 (rL2, right plot) ventral root while the positions of the red circles indicate the times of onset of activities in the left L5 (lL5) ventral root for left/right coordination in the left plot, and the right L5 ventral root for flexor/extensor coordination in the right plot. The lines within the polar plots are a graphic representation of the *r* value, while the dashed circles represent the p values ($p = 0.05$ and $p = 0.001$ for small and large circles, respectively). There is a strong synchrony apparent for onset of bouts of activity for ipsilateral

roots, while the onset of the contralateral activity is relatively variable, and is restricted to periods of ipsilateral quiescence.

Figure 5. Individual bouts of GRP_{18-27} -induced activity often exhibit fast frequency rhythmic discharges in ipsilateral ventral roots.

A: Raw ventral root activity induced by cervically applied by GRP_{18-27} (510 nM) at a fast time scale. The onset of each fast burst of activity (at ~6 Hz) is marked by the coloured vertical lines for both the L5 and L2 traces. B: Polar plot resulting from plotting the onset times of fast bursts, demonstrating that although there is a high degree of variability, the fast frequency rhythmic discharge is relatively synchronized between the two ipsilateral ventral roots. The *r* value corresponds to the degree of correlation also indicated by the line within the polar plot, while the p values are also indicated graphically as the dashed circles ($p = 0.05$ and $p = 0.001$ for small and large circles, respectively).

Figure 6. Intraspinally mediated state-dependent voltage threshold hyperpolarization occurs in neonatal rat lumbar motoneurons.

A: Raw lumbar ventral root activity (upper traces) induced by cervically applied GRP_{18-27} (250 nM) and a simultaneous voltage clamp recording from a motoneuron in the left L5 segment (last two traces at the bottom). From a -60.0 mV holding potential, two identical series of depolarizing voltage steps (+2.0 mV increments; bottom trace) were delivered to the motoneuron for determination of its V_{th}. The first voltage step and the smallest step inducing the first fast inward current are indicated by numbers in circles. B: Membrane current records (upper traces) corresponding to the minimum voltage step able to induce a fast inward current

(V_{th}, indicated by arrows). While V_{th} during the quiescent period was -38.0 mV (left, black trace), this value changed to -44.0 mV during rhythmic activity (right, red trace). This represents a -6.0 mV hyperpolarization of V_{th} associated with the ipsilateral rhythmic activity.

Figure 7. Spontaneous firing during rhythmic motor output shows reversible state-dependent hyperpolarization of motoneuron voltage threshold.

A: Raw activity from the fifth lumbar ventral root on the right side of the spinal cord (rL5 ventral root, upper trace) induced by cervically applied GRP_{18-27} (250 nM) and a simultaneous current clamp recording from a motoneuron in the corresponding lumbar segment (rL5 motoneuron, bottom trace). This motoneuron fired spontaneously throughout the recording without the injection of stimulating current. A few action potentials were present during periods of ventral root quiescence whereas bouts of repetitive firing were observed in association with the occurrence of ipsilateral ventral root bursts throughout several episodes of rhythmic network output, as the episode illustrated here. The V_{th} values from the first spike prior to, during and after the occurrence of the burst are indicated in B. While the V_{th} of the spontaneous spike on the quiescent period prior to the burst was -40.9 mV (left, dotted line), this value changed to -44.6 mV during the rhythmic activity (center, red dotted line). This change in V_{th} was reversible because the V_{th} returned to -39.5 mV after the burst had ended (right), a value similar to the V_{th} from the ventral root quiescence conditions prior to the burst. This change represents a -3.7 to -5.1 mV hyperpolarization of the V_{th} associated with ipsilateral rhythmic activity.

Figure 8. State-dependent motoneuron voltage threshold hyperpolarization occurs in most but not all episodes of spinally-generated motor activity throughout the course of each experiment.

A: Plot of the V_{th} averages for each of 27 lumbar motoneurons. Groups of dots represent the average V_{th} from a single motoneuron under 3 different conditions: prior to GRP_{18-27} application to the cervical cord ($\dot{x}V_{th}$ Control, open circles), periods of ventral root quiescence after GRP_{18-27} application, present in between bouts of rhythmic network activity induced by the peptide ($\dot{x}V_{th}$ GRP-quiescence, black circles), and periods of bursting activity occurring in the ventral roots ipsilateral to the motoneuron ($\dot{x}V_{th}$ GRP-active, red circles). Each group of data points represents a separate experiment. Statistical significance between the GRP-active V_{th} average and either the Control or GRP-quiescent V_{th} average is indicated (*). B: Graph of the incidence of V_{th} hyperpolarization associated with ipsilateral ventral root bursting. Each bar represents a single motoneuron as in A, and indicates the proportion of trials in which state-dependent Vth hyperpolarization was observed within the course of each experiment whereas the remaining percentage represents cases where V_{th} hyperpolarization might have been predicted by ongoing motor activity but a change in V_{th} was not observed.

Figure 9. Intraspinally mediated voltage threshold depolarization in neonatal rat lumbar motoneurons is associated with contralateral network bursting.

A: Raw ventral root activity (upper traces) induced by cervically applied GRP_{18-27} (250 nM) and a simultaneous voltage clamp recording from the same lL5 motoneuron in Figure 6 (bottom traces). A single bout of activity is observed in the ventral roots contralateral to the recorded motoneuron (rL5 and rL2 ventral roots) while the ipsilateral ventral roots remained

quiescent. From a -60.0 mV holding potential, three identical series of depolarizing voltage steps (+2.0 mV increments) were delivered to the motoneuron for determination of its V_{th} prior to, during and after the contralateral root burst. The first voltage step and the smallest step inducing the first fast inward current are indicated by numbers in circles. B: Membrane current records (upper traces) corresponding to the minimum voltage step able to induce a fast inward current (bottom traces, V_{th} indicated by arrows). While the V_{th} previous to network activity was -38.0 mV (left, black trace), this value changed to -36.0 mV during contralateral rhythmic activity (middle, blue trace). This +2.0 mV depolarization of V_{th} was reversible (right, black trace) once the contralateral bursting had ended.

Figure 10. Methoctramine blocks most but not all network-induced voltage threshold hyperpolarization cases associated with rhythmic activity throughout the course of an experiment.

A-C: Raw ventral root activity (upper traces on each panel) induced by cervically applied GRP_{18-27} (250 nM) and simultaneous voltage clamp recordings of an L5 motoneuron located in the right side of the spinal cord (bottom trace of each panel). Only the output from the two ventral roots ipsilateral to the neuron (rL2 and rL5 ventral roots) and one contralateral root (lL5 ventral root) are shown. As in previous examples, motoneuron V_{th} was determined by application of a series of depolarizing steps from a -60.0 mV holding potential until the first fast inward current is observed (numbers in circles). All panels represent different moments of a single experiment and with the same neuron. A: After initiation of rhythmic activity and in absence of drugs into lumbar segments of the cord, a -2.0 mV V_{th} hyperpolarization of this motoneuron was observed in association with ipsilateral ventral root activity (-52.0 mV during

bursting (red) *vs* -50.0 mV during ventral root quiescence, (black)). B: 4 minutes after lumbar application of methoctramine (20 mM), a M_2 muscarinic antagonist, the V_{th} prior to and during ipsilateral bursting was found to be the same value during both conditions. This suggests that methoctramine blocked the network-induced changes in V_{th}. However, later in the experiment and in spite increasing methoctramine concentration to 40 mM (C), V_{th} hyperpolarization associated to ipsilateral activity was still present. Note that the reference V_{th} value in absence of ventral root activity in C (-54.0 mV) was lower than that of A and B (-50.0 mV). Motoneuron in C was held at -70.0 mV and thus more steps were required to elicit a fast inward current. Overall, these results suggest that M_2 receptors are involved in mediating V_{th} hyperpolarization associated to rhythmic activity but that their action is not required for these changes to occur.

Figure 11. Averaged voltage threshold values reflect variability in the effects of cholinergic antagonists among experiments.

A: Summary of data from 9 experiments in which methoctramine was tested in its ability to block state-dependent V_{th} hyperpolarization. Dots represent the average V_{th} of a single motoneuron under 5 different conditions: control (open circles), control after GRP_{18-27} application into the cervical compartment (GRP-quiescence; black), rhythmic activity (during GRP_{18-27}-induced activity in the ventral roots ipsilateral to the motoneuron, referred as GRP-active; red), control in the presence of methoctramine (GRP+Methoc quiescence; gray) and rhythmic activity in the presence of methoctramine (GRP active + Methoc; green). Each group of data points represents a separate experiment. Statistical significance between pairs of points

is indicated (*). B: Summary of data in which the nicotinic antagonist mecamylamine was tested (blue), otherwise as described for panel A.

Figure 12. Cholinergic antagonists reduce the incidence of motoneuron voltage threshold hyperpolarization associated to rhythmic motor output.

A: Graph of the incidence of motoneuron V_{th} hyperpolarization associated with ipsilateral GRP_{18-27}-induced motor activity, as described in Figure 8B. Each group of bars represents a single experiment in which the incidence of state-dependent V_{th} hyperpolarization prior to (black bars) and following methoctramine application to the lumbar spinal cord (gray bars) is compared. B: Graph of the incidence of state-dependent V_{th} hyperpolarization prior to and after mecamylamine administration, as in A. Both cholinergic antagonists reduce the incidence of motoneuron V_{th} hyperpolarization associated to rhythmic motor output, suggesting that M_2 muscarinic and nicotinic receptors are involved in mediating these changes.

Figure 1

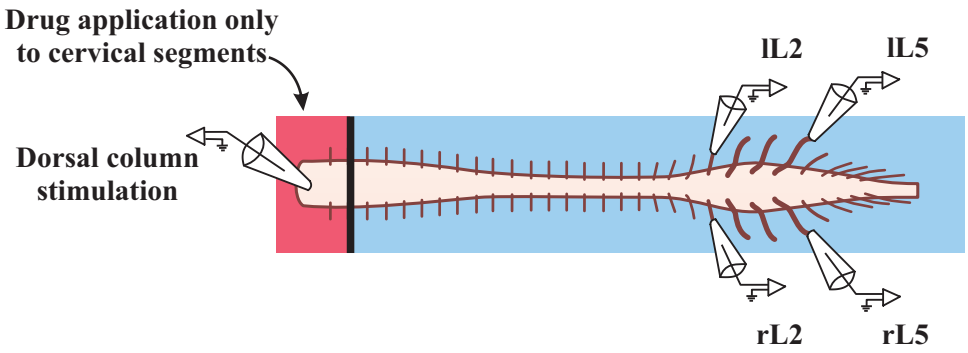

Drug application only
to cervical segments
Dorsal column
stimulation
lL2
lL5
rL2
rL5

Figure 2

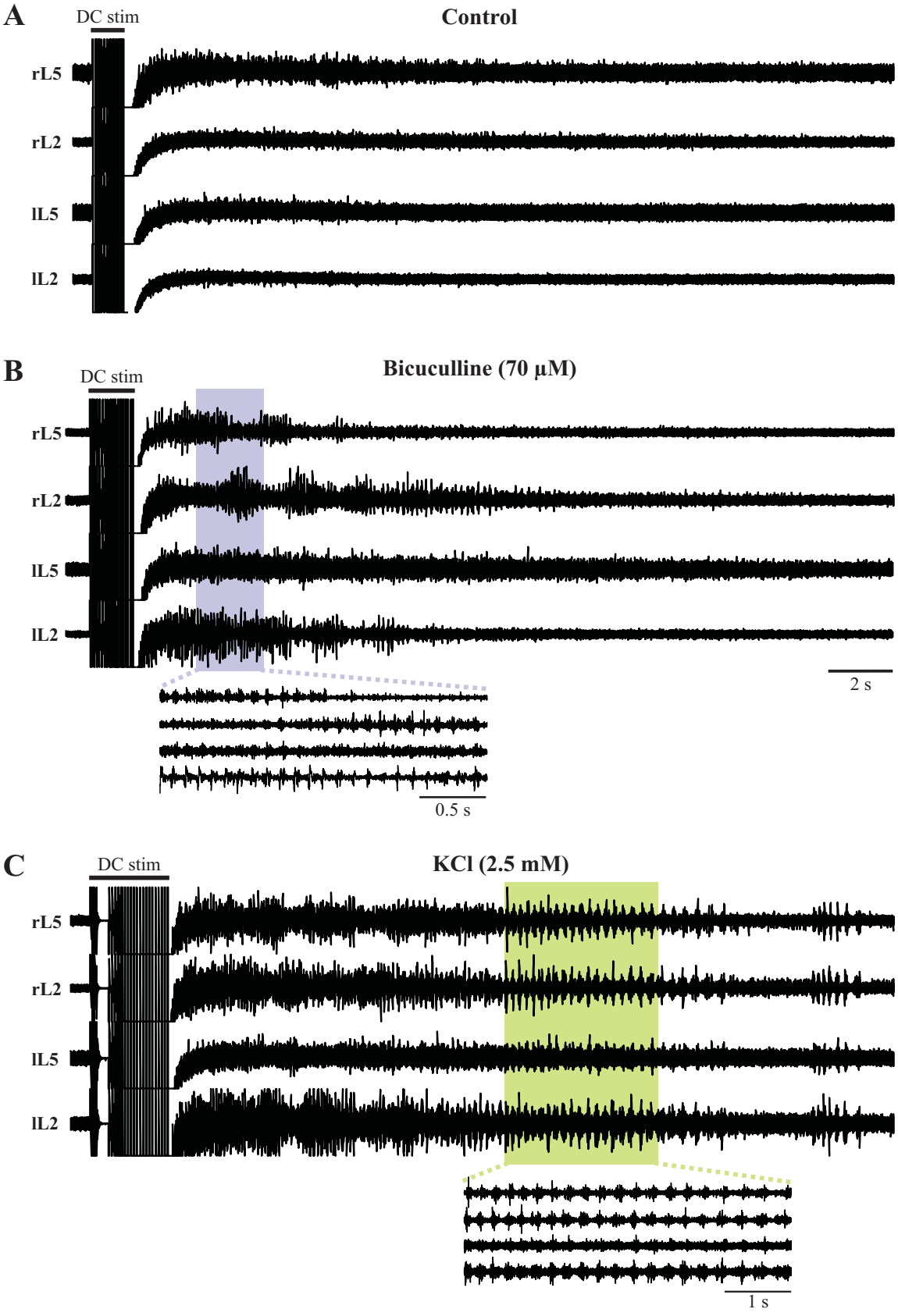

A
DC stim
Control
rL5
rL2
lL5
lL2
B
DC stim
Bicuculline (70 µM)
rL5
rL2
lL5
lL2
2 s
0.5 s
C
DC stim
KCl (2.5 mM)
rL5
rL2
lL5
lL2
1 s

Figure 3

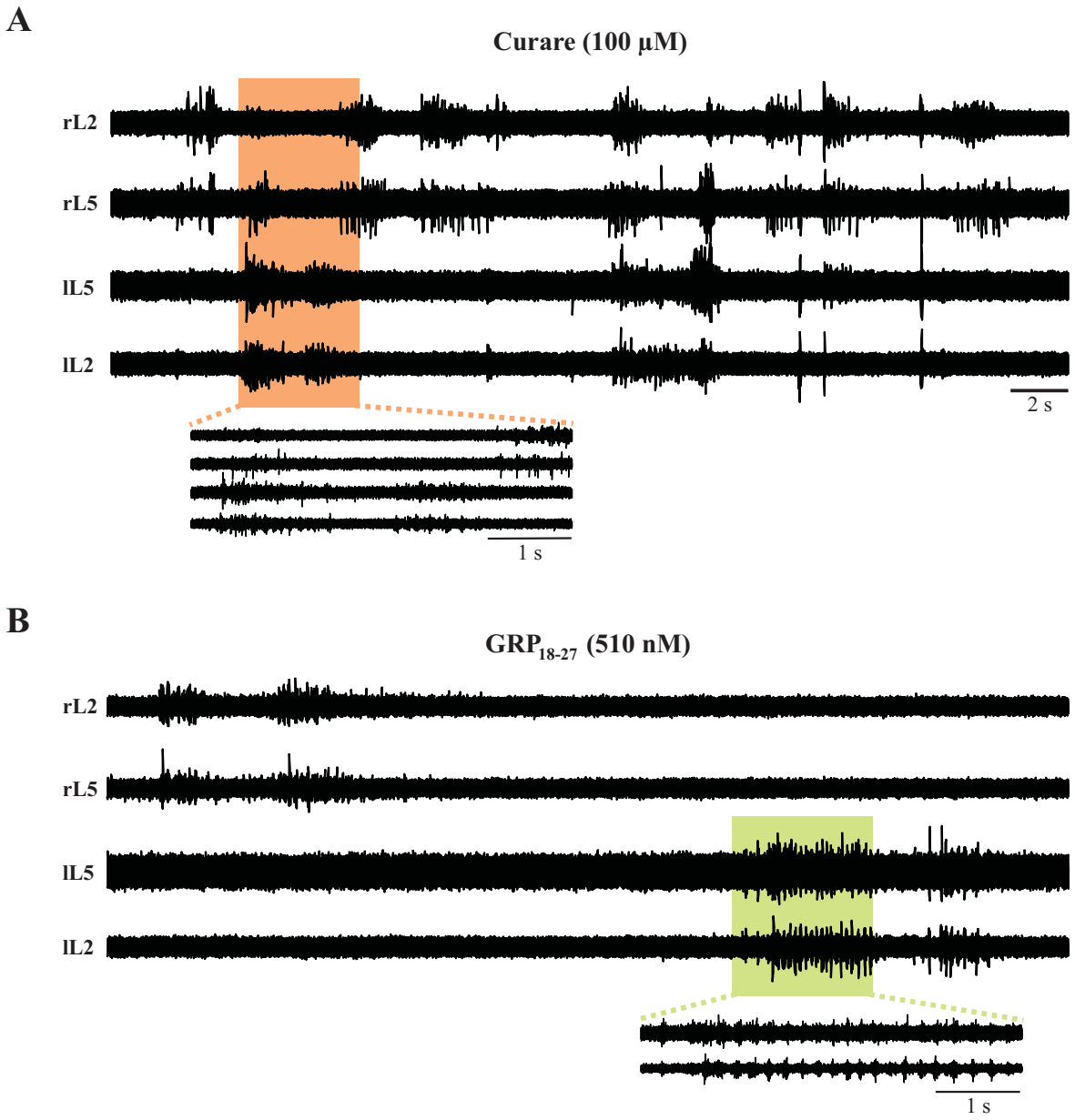

A
Curare (100 µM)
rL2
rL5
lL5
lL2
2 s
1 s
B
GRP18-27 (510 nM)
rL2
rL5
lL5
lL2
1 s

Table 1

Table 1. Effect of cervically applied neurochemicals on lumbar motor output.

Neurochemical	Concentration range (μM)	Rat age	Facilitation of fast frequency in response to dorsal column stim	Activation of spontaneous fast frequency	n
BIC	10-20	P3	-	+/-	4
	60-200	P3	++	+/-	
	100-200	P4	++	++	
	10-70	P4	+++	+	
BIC + STR	20-280	P4	++	+	4
	30-300	P1	+/-	-	
	10-70	P3	++	+	
	10-40	P4	++	+/-	
BIC + CUR	10-60	P0	+/-	-	5
	10-70	P1	+	-	
	30-50	P2	+	+/-	
	60-200	P3	++	+/-	
	20-100	P5	++	+++	
KCl	+BIC+STR	P1	-	++	4
	+STR	P3	+	+++	
	+NMDA+CUR	P4	+	+	
	alone	P4	+++	+/-	
CUR	1-3	P0	-	-	4
	3-16	P3	+/-	-	
	30-50	P4	++	++++	
	20-100	P5	-	++++	
GRP$_{18-27}$	0.1 nM-1	P5	++	++++	14
	200 nM-1	P3-P5	++	++++	

BIC: bicuculline; CUR: curare; STR: strychnine; KCl: potassium chloride; NMDA: N-methyl D-aspartate; GRP$_{18-27}$: gastrin releasing peptide receptor agonist 18-27; P0-5: postnatal day 0-5; + and -: symbols denoting the ability degree of a compound to either facilitate a fast frequency rhythmic bursting elicited by dorsal column stimulation of the first cervical segment of the spinal cord, and/or to produce spontaneous bursting activity; n: number of experiments per condition.

Figure 4

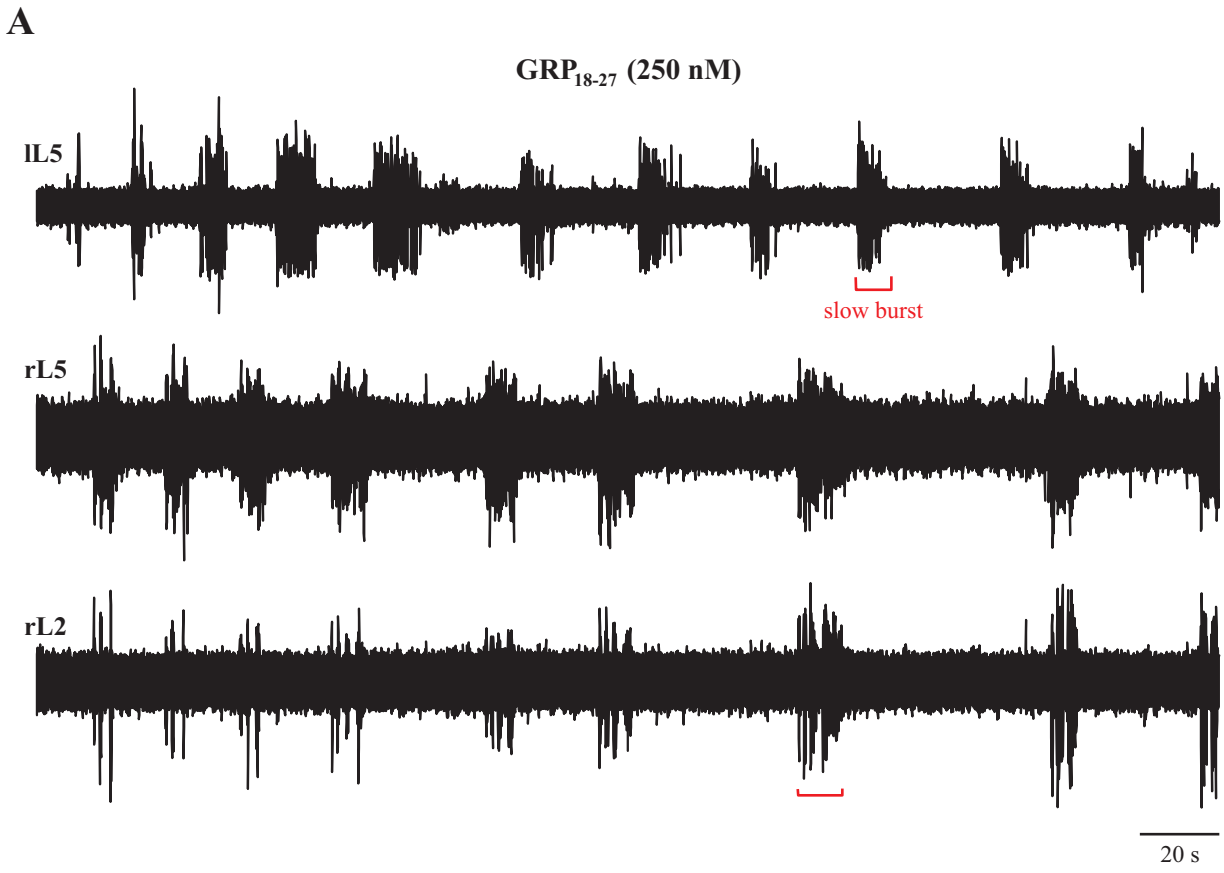

A
GRP18-27 (250 nM)
lL5
slow burst
rL5
rL2
20 s
B
LEFT vs RIGHT
slow bursts
90
180
0
270
r = 0.66
p = 0.05, cR = 0.60
p = 0.001, cR = 0.84
L2 (flexor) vs L5 (extensor)
slow bursts
90
180
0
270
r = 0.99
p = 0.05, cR = 0.69
p = 0.001, cR = 0.93

Figure 5

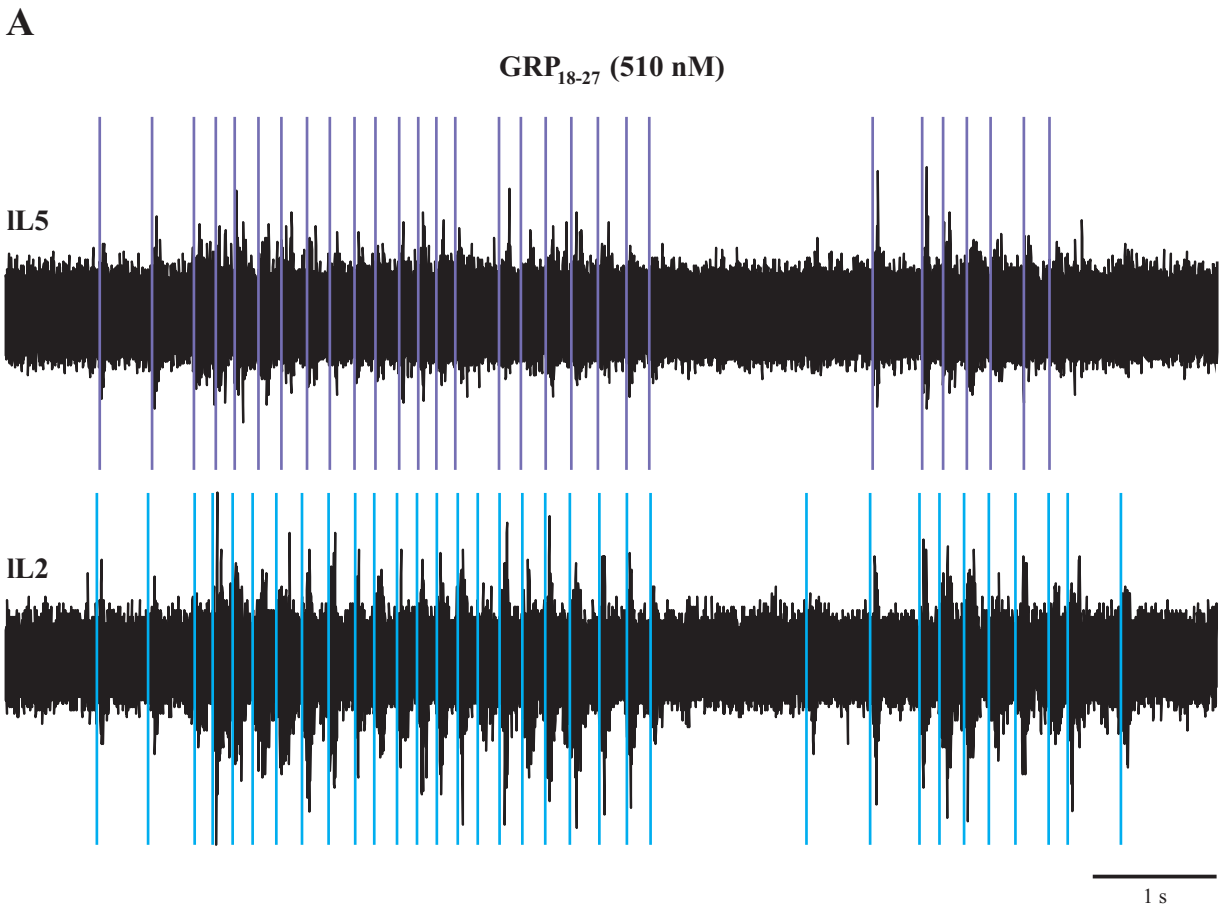

A
GRP18-27 (510 nM)
lL5
lL2
1 s
B
L2 (flexor) vs L5 (extensor)
fast bursts
90
180
0
270
r = 0.84
p = 0.05, cR = 0.34
p = 0.001, cR = 0.51

Figure 6

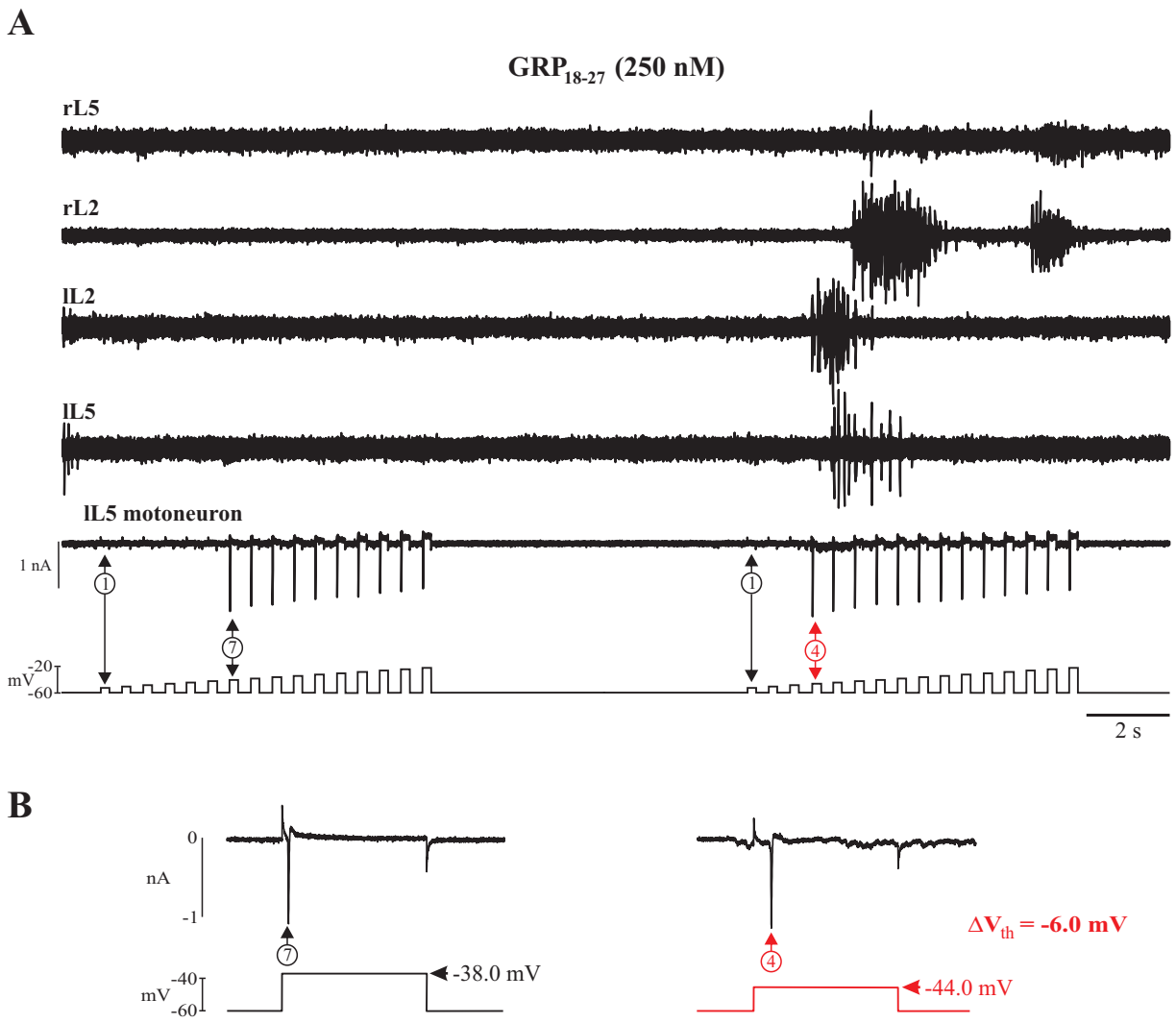

A
GRP18-27 (250 nM)
rL5
rL2
lL2
lL5
lL5 motoneuron
1 nA
mV
-20
-60
2 s
B
0
nA
-1
mV
-40
-60
-38.0 mV
ΔVth = -6.0 mV
-44.0 mV

Figure 7

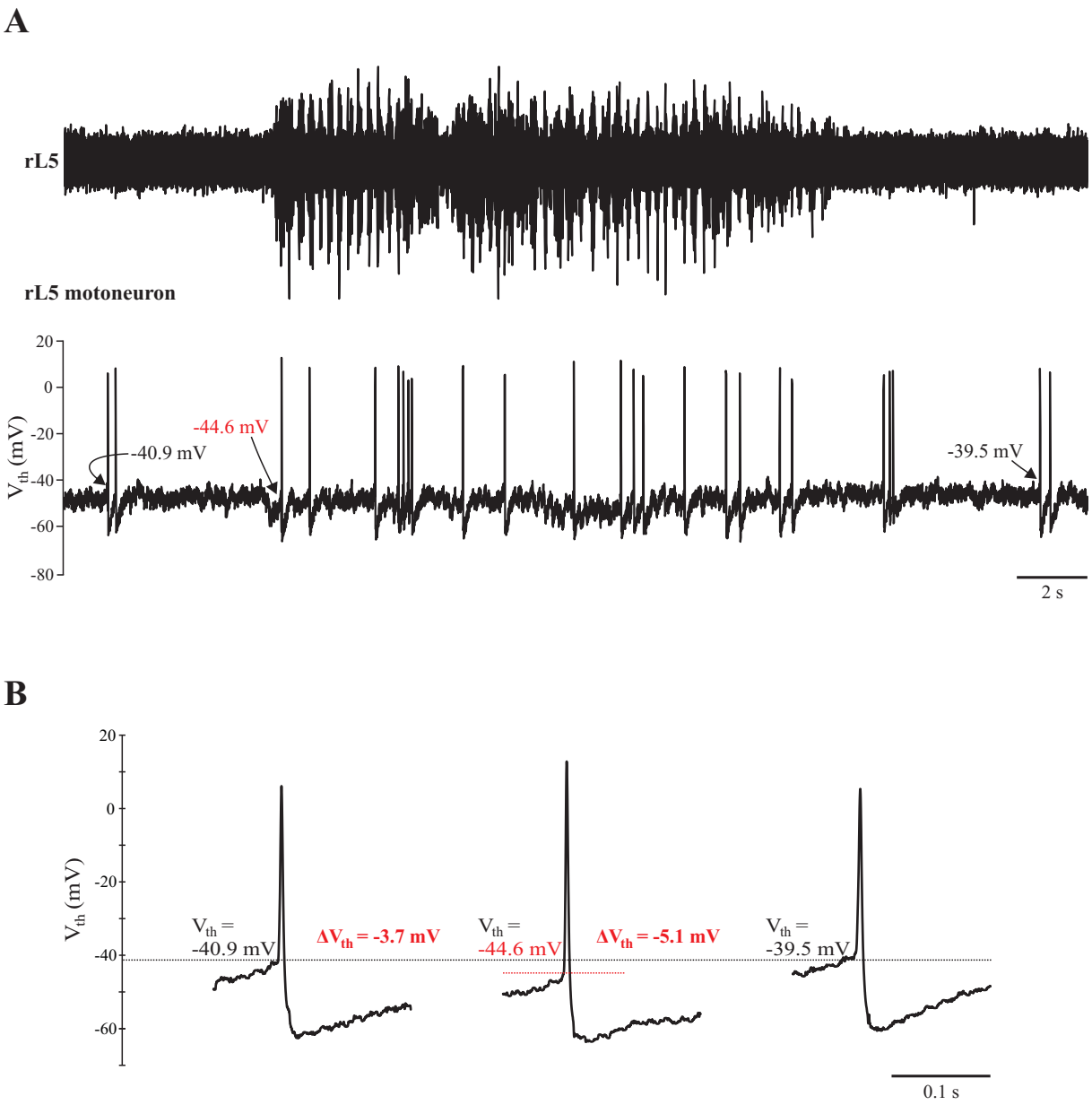

A
rL5
rL5 motoneuron
20
0
-20
-40
-60
-80
V_th (mV)
-40.9 mV
-44.6 mV
-39.5 mV
2 s
B
20
0
-20
-40
-60
V_th (mV)
V_th = -40.9 mV
ΔV_th = -3.7 mV
V_th = -44.6 mV
ΔV_th = -5.1 mV
V_th = -39.5 mV
0.1 s

Figure 8

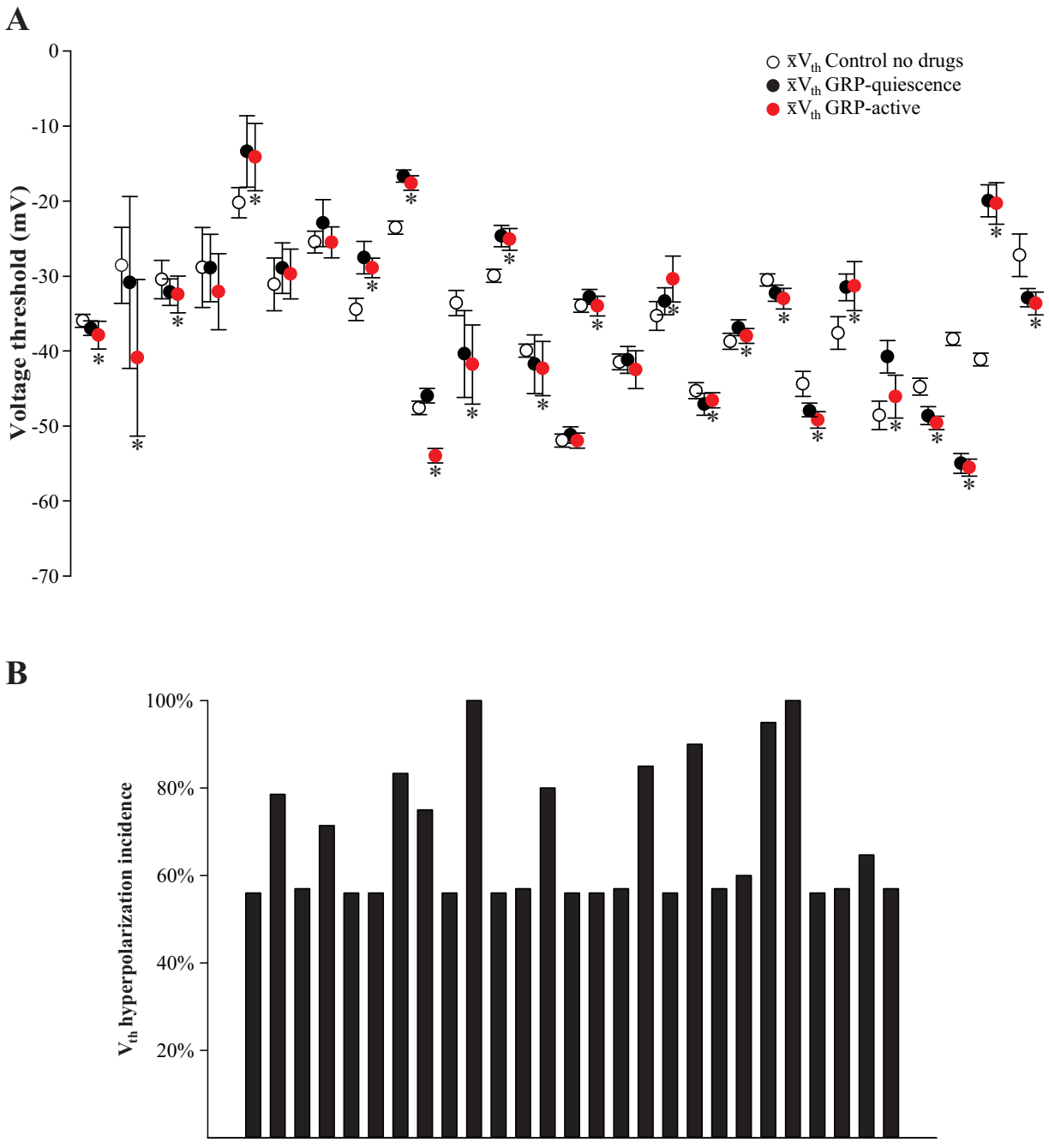

A
B
Voltage threshold (mV)
0
-10
-20
-30
-40
-50
-60
-70
$\bar{x}V_{th}$ Control no drugs
$\bar{x}V_{th}$ GRP-quiescence
$\bar{x}V_{th}$ GRP-active
V_{th} hyperpolarization incidence
100%
80%
60%
40%
20%

Figure 9

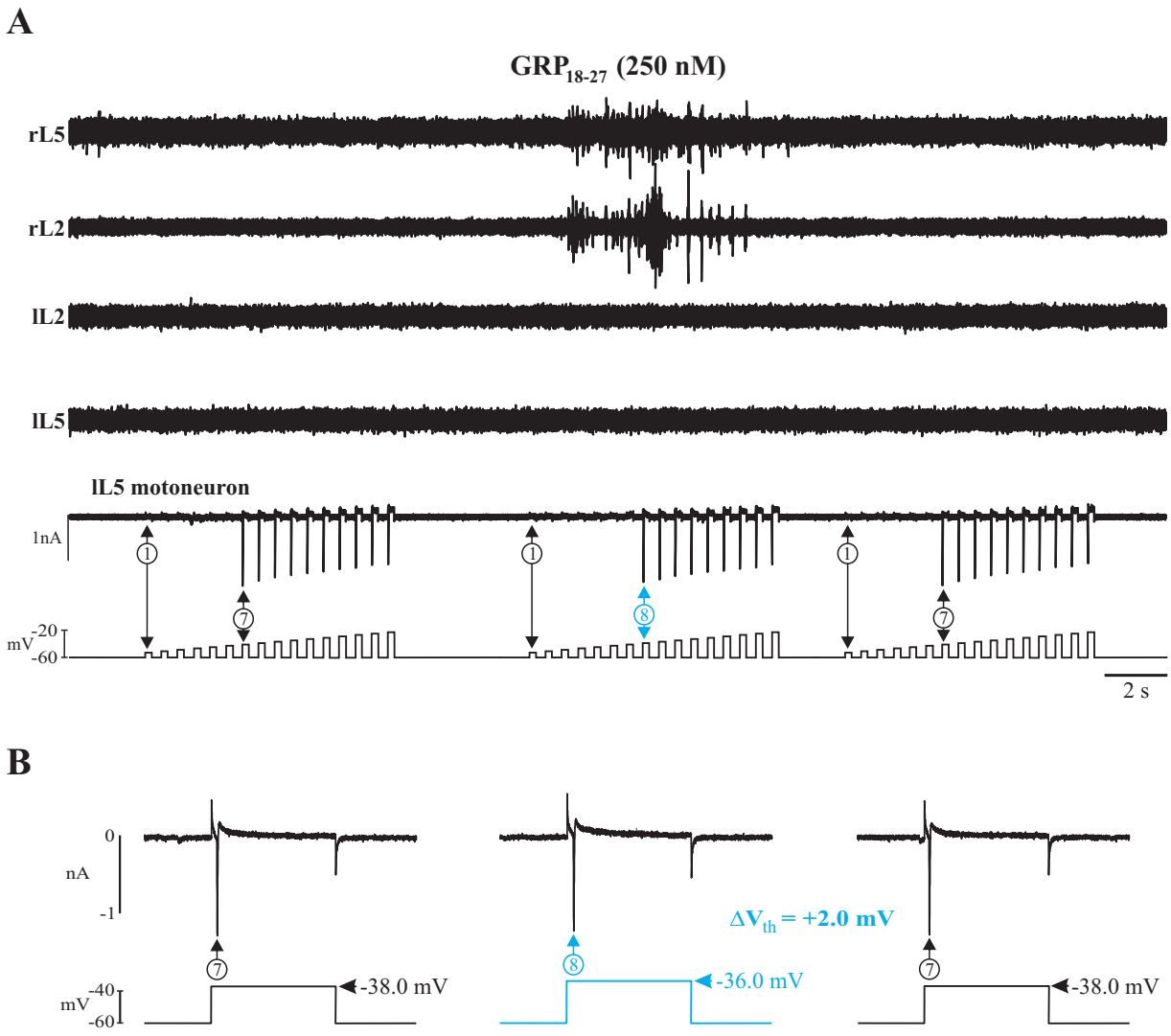

A
GRP18-27 (250 nM)
rL5
rL2
lL2
lL5
lL5 motoneuron
1nA
mV
-20
-60
2 s
B
0
nA
-1
mV
-40
-60
-38.0 mV
ΔVth = +2.0 mV
-36.0 mV
-38.0 mV

Figure 10

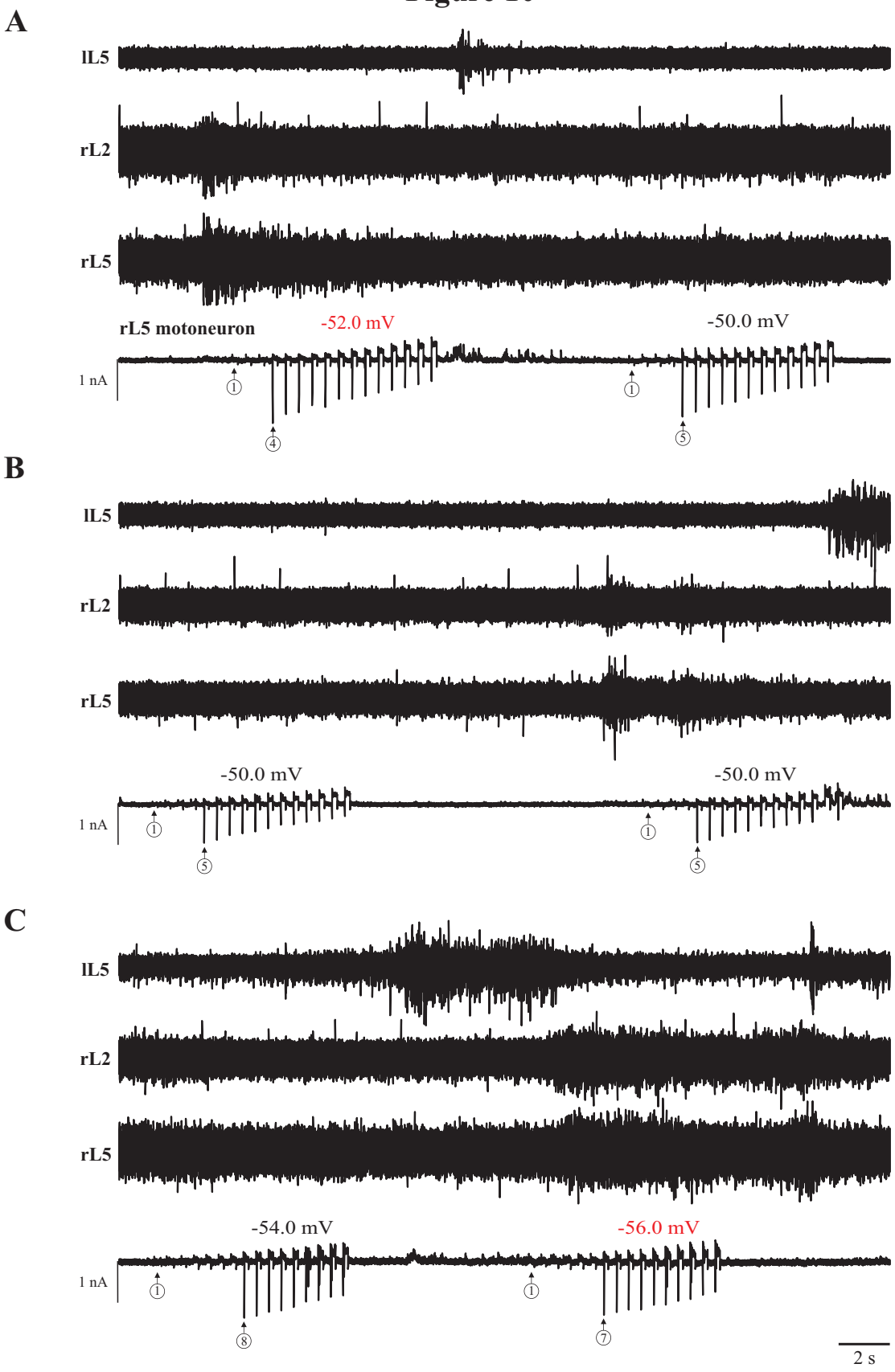

A
lL5
rL2
rL5
rL5 motoneuron
-52.0 mV
-50.0 mV
1 nA
B
lL5
rL2
rL5
-50.0 mV
-50.0 mV
1 nA
C
lL5
rL2
rL5
-54.0 mV
-56.0 mV
1 nA
2 s

Figure 11

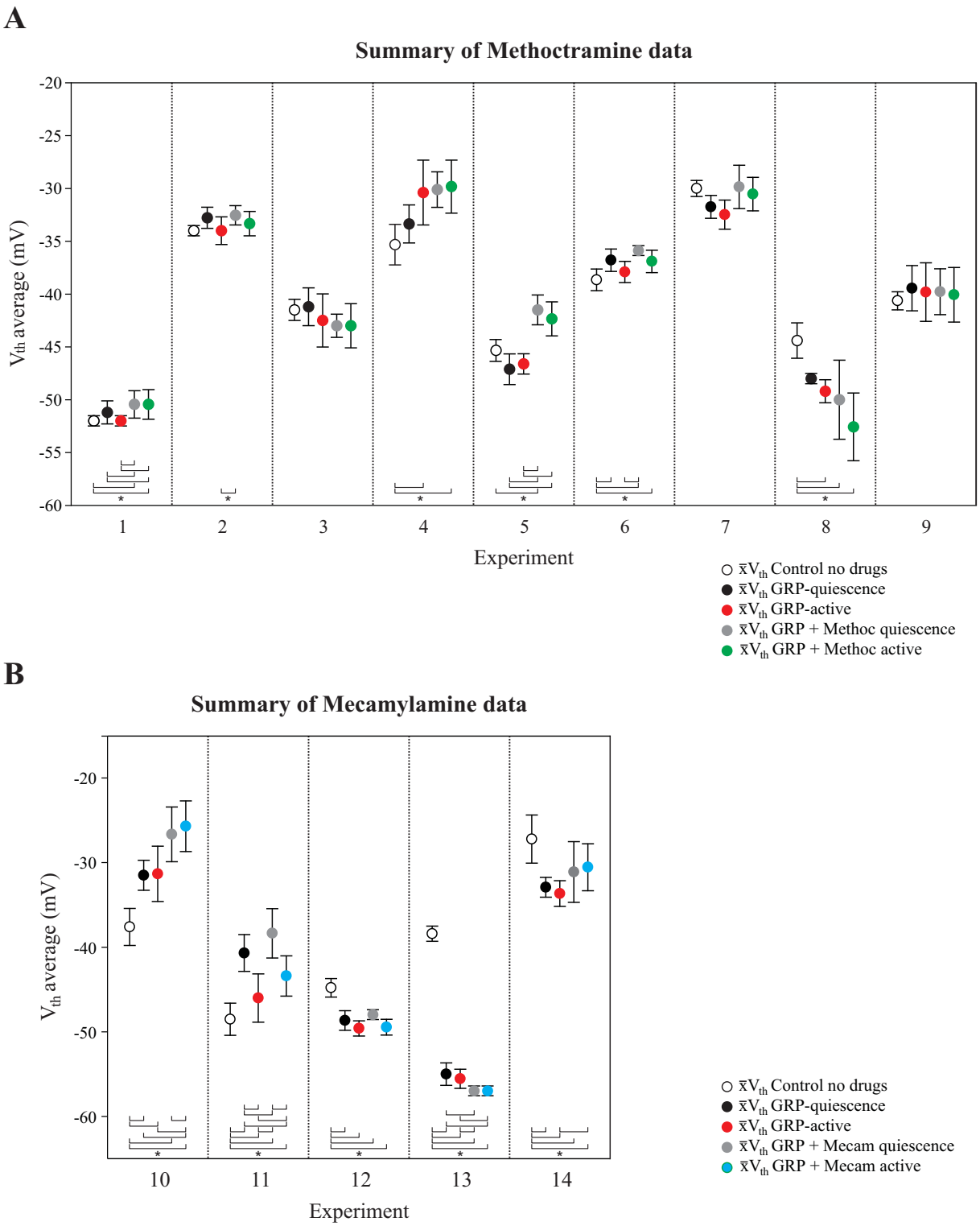
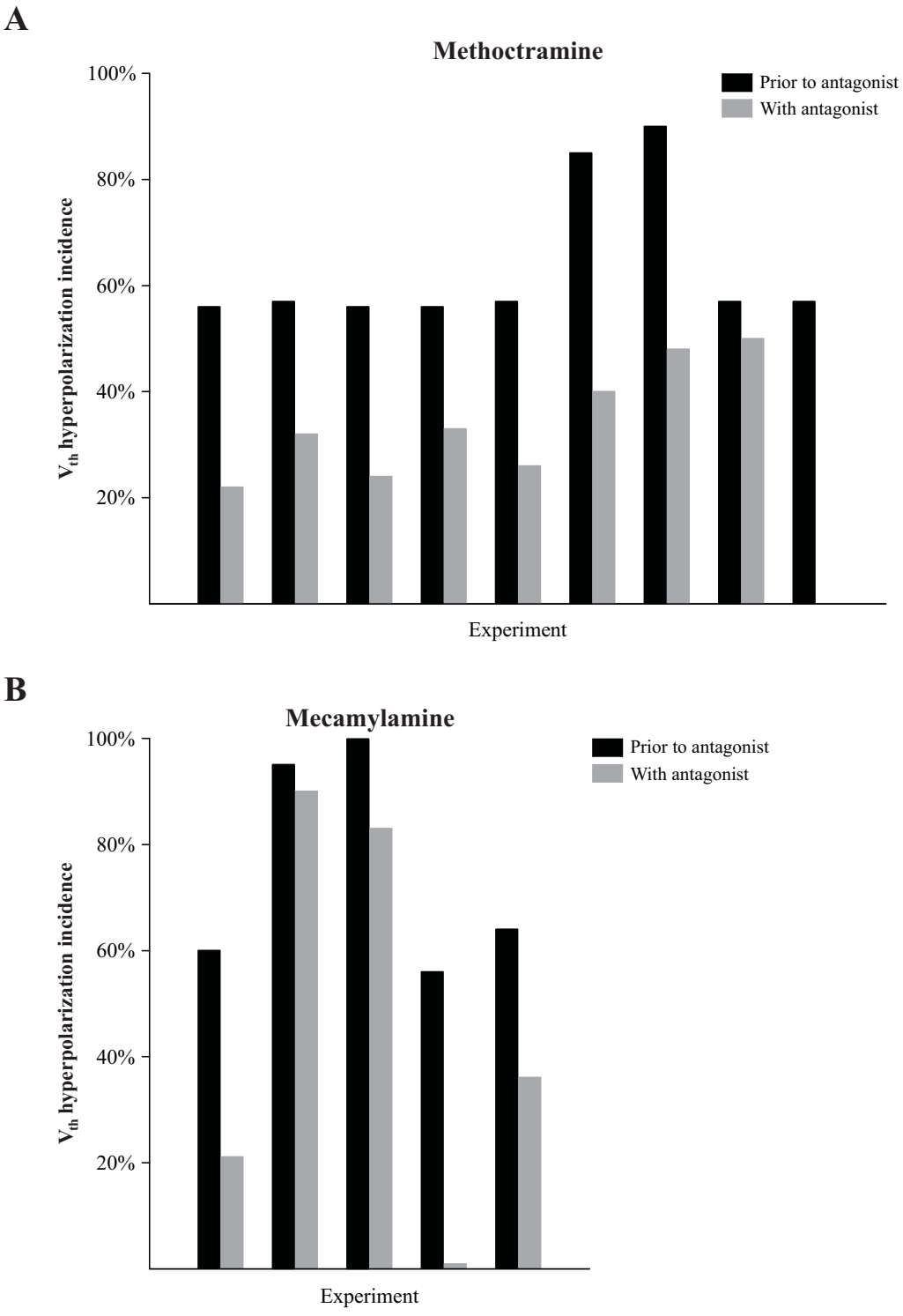

A
Summary of Methoctramine data
Vth average (mV)
-20
-25
-30
-35
-40
-45
-50
-55
-60
1
2
3
4
5
6
7
8
9
Experiment
x̄Vth Control no drugs
x̄Vth GRP-quiescence
x̄Vth GRP-active
x̄Vth GRP + Methoc quiescence
x̄Vth GRP + Methoc active
B
Summary of Mecamylamine data
Vth average (mV)
-20
-30
-40
-50
-60
10
11
12
13
14
Experiment
x̄Vth Control no drugs
x̄Vth GRP-quiescence
x̄Vth GRP-active
x̄Vth GRP + Mecam quiescence
x̄Vth GRP + Mecam active

Figure 12


A
Methoctramine
Prior to antagonist
With antagonist
100%
80%
60%
40%
20%
V_{th} hyperpolarization incidence
Experiment
B
Mecamylamine
Prior to antagonist
With antagonist
100%
80%
60%
40%
20%
V_{th} hyperpolarization incidence
Experiment

Chapter IV.

GENERAL DISCUSSION

Project goals and findings

The two studies in this thesis were designed to: (1) test the ability of ACh to alter the V_{th} of mammalian lumbar motoneurons, and (2) determine the role of the intraspinal cholinergic system in motoneuron V_{th} modulation during rhythmic network activity.

The most important finding in Paper 1 was that the modulatory effect of ACh on lumbar motoneuron V_{th} was dependent upon both the ACh concentration and network activity state. ACh induced V_{th} hyperpolarization in quiescent networks, V_{th} depolarization in spontaneously, tonically active networks and no change in V_{th} in reduced spinal networks. The most important finding in Paper 2 was that the intraspinal cholinergic system importantly contributes to motoneuron excitability regulation during rhythmic motor output by mediating state-dependent V_{th} hyperpolarization.

The relationship between cholinergic actions on V_{th} and network state was discussed throughout both manuscripts; therefore, the general discussion is reserved to present the findings of this thesis in a way to give insight into the broader picture of how distinct neuromodulatory systems regulate motoneuron excitability throughout diverse motor behaviours.

Network state and neuromodulation

Original descriptions of the V_{th} hyperpolarization during rhythmic motor output implicated descending monoaminergic pathways (Krawitz et al, 2001; Gilmore & Fedirchuk, 2004). The ability of 5-HT (Fedirchuk & Dai, 2004), NA (Fedirchuk & Dai, 2004; Tartas et al, 2010), and DA (Han et al, 2007) to directly induce V_{th} hyperpolarization in spinal motoneurons has also been demonstrated. Glutamate (Iwagaki & Miles, 2011) and ACh, as evidenced by findings in this thesis, represent additional sources for V_{th} modulation that possibly account for the V_{th} hyperpolarization occurring during scratch in spinal animals (Power et al, 2010). In addition to V_{th}, brainstem-derived monoamines have been involved in inducing resting V_m depolarization, AHP reduction and PIC facilitation that correlate well with changes observed during locomotion (see Heckman et al, 2009 for review). Although not fully characterized, ACh and glutamate exert similar effects on these properties when directly applied to spinal motoneurons (Miles et al, 2007; Iwagaki & Miles, 2011; Delgado-Lezama et al, 1997), suggesting redundancy in the ability of neuromodulatory systems to regulate motoneuron firing responses. Based on all this evidence, I constructed a conceptual schematic of the putative relative contributions of major neuromodulatory systems controlling motoneuron intrinsic properties during different motor behaviours (Figure I).

Any movement type, including locomotion and scratch in freely behaving animals, would presumably involve the activation of both descending and spinal neuronal systems. The possibility exists, however, that the proportion of modulatory actions from a certain source is higher under specific motor programs. Descending monoaminergic projections (Figure I, warm colors) then may be more actively involved in regulating neuron activity during

locomotion (e.g. perhaps accounting for ~85% of modulatory influences), whereas contributions from glutamatergic and cholinergic systems may be weaker during this motor state (perhaps accounting for the remaining 15%). Conversely, scratch, a motor behaviour relying less on supraspinal structures to be elicited experimentally, may involve relatively more intraspinal modulatory sources such as glutamate and ACh (Figure I, cold colors) than descending monoamines (e.g. putatively 85% and 15%, respectively). As outlined in the General Introduction, there is a moderate degree of overlap in the mechanisms involved in the production of hindlimb rhythmic motor behaviours such as locomotion and scratch, including the regulation of motoneuron electrical properties. Therefore, it is possible that although separate motor behaviours may rely more on a particular modulatory system to regulate motoneuron excitability, other co-activated neuromodulatory mechanisms may only modestly contribute to this regulation (Figure I, overlapping areas). Shifts in the modulatory weight between systems are discussed in more detail in the section entitled "Compensation between neuromodulatory systems".

As with rhythmic motor outputs, the production of tonic motor activity also requires modulation of motoneuron firing (Figure I, bottom circle). V_{th} hyperpolarization occurs during tonic activation of extensor motoneurons in the stance period preceding the start of fictive locomotion (Gilmore & Fedirchuk, 2004; MacDonell et al, 2015). Similarly, flexor and extensor motoneurons exhibit V_{th} hyperpolarization during the approach phase prior to the start of rhythmic fictive scratch (Power et al, 2010). In contrast, V_{th} depolarization is observed during fictive weight support in both flexor and extensor motoneurons (Power et al, 2010), an effect that seems paradoxical given the vigorous firing levels attained by extensor motoneurons during this motor behaviour. The observation that ACh can induce V_{th} hyperpol-

and depol-arization according to the network state in neonatal rats (Paper 1), raises the intriguing possibility that, in high spinal adult cats in which the V_{th} of motoneurons from separate limbs displays opposing changes during scratch (Power et al, 2010), perhaps a spinal cholinergic system is able to exert separate (and opposite) modulatory effects producing differential control of contralateral limbs. Further, in the present thesis we found V_{th} depolarization during periods of ipsilateral ventral root quiescence while rhythmic bursting occurred in the contralateral ventral roots (Paper 2), and during spontaneous activity characteristic of immature networks (Paper 1), suggesting that as weight support, other tonic motor outputs also correlate with decreases in motoneuron excitability. It is then tempting to speculate that motoneuron excitability is enhanced during tonic motor output in networks primed for the production of rhythmic motor activity in the ipsilateral limb (i.e. stance and approach; Fig. I, bottom circle overlapping areas), whereas this excitability is reduced during tonic motor output that is not related to ipsilateral rhythmic activity, despite the fact that contralateral spinal networks may or may not be engaged in rhythmic activity production (i.e., weight support and immature activity; Fig. I, bottom circle non-overlapping area). The fact that V_{th} depolarization occurs in both flexor and extensor motoneurons during fictive weight support suggests that modulators reduce the excitability of all ipsilateral motoneurons independently of the need to excite extensor (but not flexor) motoneurons during this motor output. What is then the purpose of reducing motoneuron excitability when motor output needs to be produced? The answer may lie in compensatory mechanisms put into play when a state of prolonged tonic motor network activation ensues: as V_{th} hyperpolarization facilitates the production of muscle contraction and movement generation by reducing the amount of required synaptic current to initiate motoneuron firing during rhythmic network activity, V_{th}

depolarization may potentially serve as a buffering mechanism to limit motoneuron recruitment in networks engaged in the production of sustained output, preventing excessive or detrimental muscle contraction. An example supporting this argument is provided below.

Opposing effects of neuromodulators

In addition to its excitatory effects, 5-HT has been shown to inhibit motoneuron firing by inducing V_{th} depolarization during prolonged activation of spinal motor pathways emulating sustained physical activity in humans (Cotel et al, 2013). This V_{th} depolarization was suggested to serve as a central mechanism of motor fatigue inbuilt to prevent hyperactivity of motoneurons, secure motor unit rotation, and reduce detrimental muscle activity. In the present study, ACh-induced V_{th} depolarization was observed in spinal cords displaying a type of tonic activity that is thought to correlate with network hyperexcitability, as reviewed in O'Donovan (1999). Therefore, the possibility exists that, as for 5-HT, mechanisms are put into play during prolonged motor network activity to prevent ACh-induced excessive motoneuron activation. Whether other neuromodulatory systems are involved in this mechanism is not known (Figure I, purple). Cotel et al (2013) attributed the differential effects of 5-HT on motoneuron excitability to distinct levels of endogenous 5-HT release acting at different receptor subtypes located in separate compartments of the motoneuron. They suggested that during short-lasting network activation, 5-HT released at low levels activates 5-HT_2 receptors on the motoneuron somatodendritic membrane increasing the excitability via modulation of resting V_m, AHP and PICs. After long-lasting activity, on the other hand, high levels of released 5-HT act at 5-HT_{1C} receptors in the AIS via

extrasynaptic spillover mechanisms to inhibit spiking by inducing V_{th} depolarization (Cotel et al, 2013). As in this example, V_{th} depolarization in networks programed for sustained, non-rhythmic activation (e.g., weight support, immature activity) may serve to prevent motoneuron hyperactivity. Like 5-HT, ACh seems to mediate both increases and decreases in motoneuron excitability according to the motor state. The mechanisms underlying the direction of the modulatory effects, however, may differ.

Although in Paper 1 we observed concentration sensitivity-related effects of ACh on V_{th}, it remains to be determined if distinct subcellular localization of receptor subtypes explain differences in the direction of the effects of ACh. However, this possibility seems unlikely to me because of our immunohistochemical work suggests that in contrast to 5-HT terminals, ACh terminals are located in close apposition to the motoneuronal AIS, ruling out focal *versus* spillover mechanisms underlying the opposing effects of ACh on V_{th}. Further, both muscarinic and nicotinic receptor subtypes seemed to mediate either ACh-induced depolarization or hyperpolarization of V_{th} in our preparation. Instead, our data showed that some but not all motoneurons receive cholinergic endings on their AIS, opening the possibility that a differential distribution of modulatory terminals on distinct motoneurons explain inconsistent effects of ACh on V_{th}. Alternatively (or in addition to non-uniform distribution of cholinergic terminals across a motoneuron pool), demonstration of the heterogeneity in the AIS Na^+ channel composition among mouse motoneurons (Duflocq et al, 2008) suggests that alterations in V_{th} by a single modulator may not be uniform across the pool. Duflocq et al (2008) described two populations of motoneuronal AIS: one containing $Na_V1.1$ and $Na_V1.6$ channels (distributed in proximal and distal AIS compartments, respectively) and other containing $Na_V1.6$ channels only. It is then possible that distinct effects of ACh on V_{th} may

result from modulation of separate ionic conductances in the AIS of distinct groups of motoneurons. These suggestions underscore the importance of completing our immunohistochemical studies addressing the characterization of cholinergic terminals on lumbar motoneuron AIS.

Although differences in the characteristics of cholinergic inputs to motoneurons or inter-individual variability in motoneuron properties are plausible mechanisms explaining discrepancies in the modulation of V_{th} described in Paper 1, they constitute only two possibilities among many others. Multiple putative mechanisms and functional implications exist for the opposing effects of modulators in functionally similar neurons or even within the same neuron. In a comprehensive review, Harris-Warrick & Johnson (2010) suggested that opposing actions of neuromodulators potentially: (a) result from nonspecific activation of divergent second messenger pathways and can be regarded as "evolutionary noise", provided the dominant effect is achieved, (b) constitute a mechanism to adjust the direction of the effect according to changes in the state of the network, (c) are only apparent and rather reflect modulatory action at different concentrations, voltages, time courses or locations, and/or (d) stabilize the network state, preventing it from over-modulation. As argued in Paper 1, we observed a correlation between the activity state of the network and the modulatory effect of ACh on motoneuron V_{th}. Therefore, in addition to paying attention to the characteristics intrinsic to the ACh-motoneuron synapse, it should be kept in mind that other ionotropic and metabotropic inputs importantly influence the actions of a modulator. As reviewed in the General Introduction, the state of a network can influence the action of a modulator on its target neurons by activating (or inactivating) conductances, second messenger pathways or other neuromodulatory mechanisms that interact with those altered by the modulator (see

Nadim et al, 2008 and Marder et al, 2014 for review). Interactions between neurons, modulators and networks matter the most during normal production of motor activity because, as work in molluscs indicates, in spite the existence of large inter-individual differences in intrinsic baseline properties, variability in motoneuron activity is least during relevant motor behaviour production to ensure efficacy (Cullins et al, 2015).

As suggested earlier in the present discussion, interactions between neuromodulatory systems may vary between motor behaviours. The relative contributions of modulators may be further modified by conditions in which one system is eliminated or impaired, for example after injury or disease. In Paper 2 we explored the intraspinal contributions to modulation of motoneuron V_{th} by attempting to eliminate descending modulatory influences. The next section discusses our findings in the context of short-term adaptations following acute spinal cord transections. Long-term adaptations in the cholinergic modulation of motor output are covered in the final section.

Compensation between neuromodulatory systems

Unfortunately, very few studies have investigated the interactions between different modulators. In the mammalian respiratory system, Doi and Ramirez (2010) explored the functional interactions between 3 neuromodulators: SP, 5-HT and NA, in regulating the respiratory CPG frequency. They found that although all 3 modulators exert excitatory actions on respiratory activity, tonic activation of both NA and 5-HT receptors within the respiratory CPG is required for the maintenance of a high respiratory frequency and regularity, whereas SP contributions are state-dependent. Endogenous activation of SP receptors was critical in

regulating respiration only when NA and 5-HT receptor activation was reduced or breathing frequencies were low (<1.5 Hz). This suggests that deficiencies in 5-HT and NA activity are compensated by SP to maintain breathing CPG frequency high, but otherwise SP effects are not essential (Doi & Ramirez, 2010).

In the spinal cord motor system, NA, 5-HT and DA seem to play critical excitatory roles as described for the brainstem respiratory CPG. However, their relative contributions and interactions with other modulators such as ACh and glutamate remain unexplored. In Paper 2 we used a spinal cord preparation devoid of intact supraspinal structures designed to explore the relative contribution of intraspinal systems to motoneuron V_{th} modulation. This approach was limited in that it did not allow determination of the actual contribution of intraspinal systems to modulation of motoneuron excitability because it is unlikely that these systems operate under isolated conditions during normal motor output production. Further, as in the work with spinal transected cats, it was not possible to conclude with certainty that residual monoamines may not have contributed to the observed V_{th} hyperpolarization in our preparation by monoaminergic release from sectioned axons of descending monoaminergic pathways, as well as the few intraspinal serotoninergic neurons that have been described in the spinal cord (Carlsson et al, 1963; 1964). However, it is likely that in our preparation at least a severe reduction in the input from monoaminergic pathways was achieved, allowing the assessment of whether endogenous propriospinal cholinergic sources mediated changes in V_{th} during rhythmic motor output. Our findings that ACh is not critical but importantly contributes to this modulation thus provides the first step towards the study of the interactions between spinal and descending modulatory systems in the regulation of motoneuron firing during motor behaviour. Subsequent studies using this preparation could be now designed to

determine: (1) the relative contribution of glutamatergic *vs* cholinergic inputs to V_{th} modulation, (2) the interactions between these systems and monoaminergic inputs, (3) the relative contribution of modulators in regulating other motoneuron electrical properties such as AHP, PICs, repetitive firing and action potential features, and (4) the relative contribution of modulators to regulate motoneuron excitability during distinct motor outputs such as locomotion and the GRP_{18-27}-induced rhythmic activity.

Similar to Doi & Ramirez (2010) findings, I hypothesize that ACh plays a critical role in modulating motoneuron excitability in conditions of reduced descending monoaminergic activity, such as following acute spinalization. It is clear that locomotion relies on the activation of monoaminergic projections to secure a consistent, well-coordinated CPG activity and adequate excitation of motoneurons (Figure I, warm colors). Most of this modulation may then be compensated for by propriospinal cholinergic and glutamatergic sources in acutely spinal transected animals (Figure I, cold colors). Contributions from cholinergic inputs under this condition may be stronger than glutamatergic influences (Figure I, green area > blue area). In their investigation of the effects of group I mGluR activation in locomotor-related motoneuron output, Iwagaki & Miles (2011) concluded that, although these receptors mediate V_{th} hyperpolarization, their net effect is inhibitory due to reductions in Na_T current peak density. Furthermore, they reported that in spite of their ability to modulate motoneuron output when stimulated, endogenous group I metabotropic glutamatergic receptors are not normally activated during *in vitro* locomotor-like activity. Endogenous cholinergic activity, on the other hand, has been shown to clearly increase motoneuron output during this motor output (Miles et al, 2007; Jordan et al, 2014). Despite the fact that the cholinergic system may play a compensatory role in the absence of descending monoamines, it is possible however,

that state-dependent changes in all motoneuron electrical properties may not be equally compensated in these conditions. Some properties such as PICs for example, may rely more in monoaminergic receptor activation than V_{th} or AHP (Figure I, note that ↑PICs are located in an area of mixed yellow and blue backgrounds, far from green background). Although their actions are redundant, not all modulators affect all motoneuron active properties described so far; for example, in Paper 1 we found that although ACh could induce V_{th} hyperpolarization, its actions on PICs were inhibitory independently of the motor state (Figure I, ↓PICs on green background). Although a more detailed analysis is needed to determine the specific modulatory actions of ACh on the Ca^{2+} and Na^{+} currents mediating PICs, it is likely that cholinergic inputs do not affect these currents in the same extent and direction to that exerted by monoamines. Further, it must be considered that the effects of multiple modulators might combine to produce a net modulatory effect on motoneuron properties (discussed below).

Interactions between neuromodulatory mechanisms in the spinal cord

In their study of locomotor-activated spinal neurons in transgenic mice, Dai et al (2009) found that the effects of 5-HT and ACh on the V_{th} of these neurons were additive, so that increasing levels of V_{th} hyperpolarization ensued following subsequent application of these two modulators. 5-HT and ACh application order did not alter the ability of each modulator to hyperpolarize V_{th}, suggesting that they act by different mechanisms to attain the same effect on V_{th}. Provided the ability of these modulators to summate their actions on V_{th} also exists in spinal motoneurons, it can be suggested that in contrast to brainstem-intact animals, the degree of V_{th} hyperpolarization achieved during motor output in spinalized

preparations may be limited given the strong reduction in monoaminergic input sources. It is thus possible that, although ACh compensates motor-dependent changes in V_{th} upon reductions in monoaminergic activity in individual motoneurons, the extent to which these changes occur may not be equal to that when monoamines are present. To test this hypothesis, experiments could be designed to record individual motoneurons during motor output prior to and following transections separating the brainstem from the spinal cord. The fictive cat scratch preparation or the GRP-$_{18\text{-}27}$-induced motor output in the neonatal rat preparation are both suited for this assessment because these rhythms can be readily elicited following acute spinalization without pharmacological manipulation of lumbar spinal segments. Using the same neuron as control, the amount of V_{th} change during motor output could then be compared in conditions prior to and following spinalization. The neonatal rat preparation would add the advantage of testing blockers of different modulators to assess the contributions from distinct systems to the control of V_{th} during motor behaviour under each condition.

It should be noted, however, that elucidation of the net effect on neuron excitability resulting from the combined actions of modulators is not a simple task. For example, independently of their additive effects on V_{th}, 5-HT and ACh have opposite actions in the R_{in}, I_h and AHP of locomotor-activated neurons (Dai et al, 2009). Consequently, the amount and direction of change in different membrane properties result from the proportion of 5-HT and ACh surrounding these neurons with varying effects on their overall excitability and firing behaviour. The modulatory milieu, determined by the dynamic balance between different systems actions at a point in time, constitutes a critical factor influencing the function of neurons and networks as well as their responsiveness to perturbations in other preparations such as the crustacean STG (Derjean et al, 2003; Peck et al, 2006; Nadim et al, 2008). In

addition to using separate mechanisms, neuromodulators can putatively converge in regulating specific conductances via interactions at the level of receptors and intracellular transduction pathways. For example, muscarinic cholinergic receptors predominate in monomer form in the absence of ligand binding but di- or oligo-merize upon agonist or antagonist binding (Borroto-Escuela et al, 2012). This oligomerization can occur with identical receptors (M_2-M_2, for example), with receptors from the same family (M_1-M_2, for example) and interestingly, with receptors from other families (M_4-5-HT_{2C}, NAα1 or DA_2, for example). Unfortunately, the functional implications of homo- and hetero-dimerization of muscarinic ACh receptors is poorly understood at present (Borroto-Escuela et al, 2012). Additionally, modulators are well known to exert cooperative actions when co-released from the same terminals. In a variety of neuronal systems, muscarinic responses are invariably preceded by glutamate-mediated events, presumably due to co-release of glutamate from the same cholinergic terminals (Brown, 2010). From the cholinergic inputs to spinal motoneurons, co-expression of glutamatergic markers in C-boutons originating from Pitx2+ V0 interneurons was ruled out (Zagoraiou et al, 2007). However, the synapses formed between motoneurons have been shown to co-release ACh and glutamate (Nishimaru et al, 2005) and thus represent a putative co-modulatory source to these neurons. As argued in Paper 2, it is possible that complementing ACh actions, glutamate was also involved in mediating V_{th} hyperpolarization during rhythmic motor output in preparations lacking intact supraspinal modulatory projections. This opens the possibility that a cooperative action between glutamate and ACh, perhaps released from the same terminals, is needed to fully compensate deficiencies in monoaminergic action.

All organisms, from simple to complex, utilize numerous amines, peptides, gases and other molecules to shape their neuronal behaviour. These modulators can interact with each other at practically every level of neuronal transmission, either hand-in-hand or following a hierarchy of metamodulatory mechanisms. Further, modulators interact with their target networks and its neuronal components to, again, influence and be influenced by them. For these reasons, I believe that building a complete picture of the modulatory mechanisms determining the functioning of a neuronal network is an intimidating but very interesting and worthwhile task. In the mammalian spinal motor network, the first steps toward this understanding have been taken; future work will clarify the workings of modulatory mechanisms during both normal and impaired motor behaviour.

Putative ionic mechanisms of cholinergic modulation of voltage threshold

The idea of modulatory systems routinely recruited to rapidly reconfigure neuron excitability to match behavioral demands through modulation of V_{th} is a fairly new concept, essentially originated from Krawitz et al (2001) work in mammalian motoneurons. Given its relative novelty as a described mechanism, changes in V_{th} have remained unrecognized in several studies in which the modulatory effects of ACh on motoneuron membrane properties have been assessed (see for example, Haji et al, 1996; Figs. 1 and 2: a reversible ACh-induced V_{th} depolarization is observed, and Fig. 4: ACh-induced V_{th} hyperpolarization is observed). By exploring the contribution of cholinergic influences in the control of spinal motoneuron V_{th}, findings in this thesis advance our understanding of the modulation of neuronal excitability during movement.

In the General Introduction and throughout both manuscripts, the potential mechanisms of V_{th} hyperpolarization were discussed and therefore will not be presented here in detail. Most of these mechanisms have considered modulation of Na^+ channels in the AIS and thus here I present a brief review of a particular K^+ conductance mediated by M-type channels that, to my opinion, has not been properly recognized as a possible target of modulation during motor-induced V_{th} hyperpolarization.

The M-current is a time- and voltage-dependent K^+ current that slowly activates when a neuron is depolarized toward the V_{th}, stabilizing the V_m to hyperpolarized values and reducing membrane excitability (Brown & Passmore, 2009). This current is small in amplitude (<50 pA), but given its unique characteristics of sustained activation and absence of inactivation at values close to the V_{th}, it has a major impact on neuronal excitability. Since its discovery in 1980 in sympathetic ganglion neurons, this current was named "M" because of its suppression by muscarinic receptor activation (Brown & Adams, 1980). M-current suppression results in V_m depolarization and increased probability of action potential firing; therefore, it has been suggested that this current serves as a "brake" of repetitive firing discharges (Delmas & Brown, 2005; Brown & Passmore, 2009). The muscarinic receptor subtypes mediating M-current inhibition appear to depend on cell-type, mainly involving M_1 and to less extent M_2 receptors (Brown & Yu, 2000). Although their ability to suppress or enhance the M-current varies in distinct cell types, other receptors have been shown to alter this current, including 5-HT_{2C}, β-NA, group I metabotropic glutamatergic receptors, SP, hormone, opioid and purinergic receptors (Brown &Yu, 2000). Some of these receptors activate $G\alpha_q$ and/or $G\alpha_{11}$ G-proteins that initiate PLC-β activity thereby decreasing membrane phosphatidylinositol-4,5-biphosphate (PIP_2) concentration levels by 90% to inhibit M-current,

given that PIP_2 is required for these channels to open (Delmas & Brown, 2005). Because at normal resting concentrations of PIP_2, M-channels are not fully open, considerable room for increasing channel activity exists to explain modulator-induced enhancement of M-currents (Delmas & Brown, 2005). The M-channel typically consists of a heterotetrameric structure formed by KCNQ2 and KCNQ3 subunits of the Kv7 family of K^+ channels (Kv7.2 and Kv7.3, respectively) (Brown & Yu, 2000; Delmas & Brown, 2005; Brown & Passmore, 2009). Mutations in Kv7/KCNQ genes are associated with cardiac arrhythmias and a familial epileptic syndrome in humans, underscoring their involvement in cell excitability regulation (Brown & Passmore, 2009).

Although classically associated with regulating synaptic integration in somatodendritic areas, M-channels were recently shown to concentrate at higher densities in the AIS, where they could be critically regulating the action potential V_{th} (Brown & Passmore, 2009; Kress & Mennerick, 2009). This was in fact demonstrated by Shah et al (2008) in rat hippocampal pyramidal neurons. To assess the specific contribution of AIS M-channels to neuron excitability, this group designed an AnkG-binding peptide (ABP) having an identical amino acid sequence unique to the Kv7.2/7.3-AnkG binding motif. By using ABP in the internal solution of the recording patch clamp electrode, they found that this peptide effectively diffuses to the AIS of the neurone and competes with Kv7 subunits for binding AnkG, thereby dissociating the channels from the AIS membrane. Disruption of the targeting of AIS Kv7 subunits resulted in steady resting V_m depolarization, spontaneous spiking, and remarkably, V_{th} hyperpolarization (mean -7.8 mV). Subsequent bath application of (or previous treatment with) a potent M-channel inhibitor, XE991, did not have further effects on V_{th}, indicating that somatic Kv7 channels unaffected by ABP did not contribute to the observed V_{th}

hyperpolarization (Shah et al, 2008). Neither ABP nor XE991 had effects on action potential height, half width or rate of rise, suggesting that Na^+ and K^+ channels underlying spiking were unaffected. Further, they used computer modeling to corroborate their experimental findings and found that, as shown by anatomical studies, a Kv7 soma:AIS ratio between 1:2 and 1:5 is required to reproduce the observed changes (Shah et al, 2008).

Interestingly enough, KCNQ2 subunits have been recently shown to be present in the AIS of all α-motoneurons of adult mice (Duflocq et al, 2011). Distribution of these subunits along the AIS is uniform, closely matching that of AnkG. I believe that given this amount of evidence, investigation of the contribution of the M-channel conductance to the control of motoneuron V_{th} using pharmacological tools such as ABP and computational modeling is worth pursuing. If AIS M-channels result to mediate changes in spinal motoneuron V_{th}, this mechanism would provide a nice fit to observations on this thesis that muscarinic receptors mediate V_{th} hyperpolarization both when activated exogenously (by pharmacological manipulation, Paper 1) or endogenously (by motor network activation, Paper 2). If true, this finding would have a strong impact on the mechanisms of V_{th} hyperpolarization associated with rhythmic motor output production because the channels and transduction pathways involved in this effect remain obscure until now.

Brief comment on the mechanisms of itch and scratch

Findings in the present thesis can also speak to an area of research with focus on the mechanisms of itch perception and its intricate relationship with the processing of pain signals. Itch is a common sensory experience usually abolished by briefly scratching the

irritated area. However, itch can also emerge as a common symptom of a wide variety of skin and system diseases such as chronic renal disease, cholestasis, multiple sclerosis, brain tumours, nerve injury and post-herpetic neuralgia, for example (Yosipovitch et al, 2003; Misery et al, 2014). Also, several drugs to treat diseases such as hypertension or pain, induce persistent itch as a side-effect (Misery et al, 2014). In all these conditions, the peripheral (pruritoceptive) and/or central (neurogenic) components of itch are altered so that local scratching exacerbates the sensation instead of relieving it, producing a distressing state in humans that can severely impair the quality of life. The lack of a specific anti-pruritic treatment (Leslie et al, 2015) has led researchers in this field to develop several animal models to investigate the mechanisms of itch perception, most using transgenic mice as primary models. Most test using these models utilize behavioural responses such as hindlimb scratching for itch and forelimb wiping or paw withdrawal for pain for example, as indicators of perceived sensations following administration of pruritogenic and nociceptive stimuli (Kuraishi et al, 1995; Spradley et al, 2012). Although instrumental for advances in this field, these approaches are intrinsically limited for the pharmacological and electrophysiological characterization of the spinal and supraspinal neuronal circuitries processing the itch signal. Few studies have utilized cat or rat *in vivo* pentobarbital-anesthetized preparations to perform extracellular recordings of specific dorsal horn spinal neurons (reviewed in Carstens & Akiyama, 2014). However, as discussed in the General Introduction, these preparations are inadequate for the pharmacological manipulation of specific neuronal subsets, as well as for the investigation of neuronal responses during relevant behaviour (such as motor output production) as opposed to a “depressed” (or at least altered) anesthetized state. This highlights the need of an *in vitro* preparation conferring the possibility to study and manipulate neurons

involved in the itch pathways in relation to the production of an itch-specific motor response such as scratch.

Although originally designed to investigate the mechanisms of motor output modulation, the neonatal rat spinal cord *in vitro* preparation introduced in Paper 2 meets the requirements for the study of spinal itch pathways enlisted above. Our evaluation of the putative transmitters capable of triggering a scratch-like motor output in neonatal rat spinal cords was the first to show to my knowledge, that a molecule involved in relying the itch signal in the spinal cord, GRP, complies with mechanisms preserved across several motor behaviours such as rhythmic ventral root output production and state-dependent modulation of motoneuron excitability. Although not strictly matching the hindlimb nerve activity pattern of scratch as described in cats (Rossignol, 1996) or the scratching hindlimb movement as observed in unrestricted neonatal rat pups (Vasquez-Dominguez & Fedirchuk, unpublished observations), the GRP_{18-27}-elicited rhythmic ventral root output could be explored as a motor signature of itch signaling in the neonatal rat *in vitro* spinal cord preparation. Of course, this would require a more detailed characterization of the motor responses to pruritogenic stimuli in neonatal rats. For example, a comparison among different itch-induced responses is necessary, including: (1) the scratch muscle activation patterns in neonatal pups in response to diverse pruritogenic agents applied to the skin, (2) the motor response to intrathecally applied GRP_{18-27} in neonatal rat pups and (3) the ventral root/nerve activity patterns elicited by GRP_{18-27} in the *in vitro* preparation. Also, a comprehensive characterization of the *in vitro* motor responses to focal application of GRP_{18-27} to selected dorsal horn areas in distinct spinal cord segments and across a broader range of age in rats (and mice) would be helpful. Further, this preparation allows the exploration of whether nociceptive-specific transmitters elicit forelimb

wiping-like responses so that both pain and itch mechanisms could be compared in the same preparation, as is a standard of studies in this field. In sum, our novel preparation offers the potential of investigating the mechanisms involved in both itch and scratch, and thus brings together two fields of research (sensory and motor) that, surprisingly, have remained largely isolated.

Final thoughts on the relevance of findings in this thesis: chronic spinal cord injury mechanisms

The existence of redundancy in the ability of diverse neuromodulatory systems to regulate membrane motoneuron properties, as well as the prevalence of state-dependent changes in these properties across different motor outputs, suggests that regulation of the excitability of spinal motoneurons is a critical component of movement generated by spinal networks. Following spinal cord injury, the descending commands required to initiate these movements as well as to modulate motoneuron output are lost or impaired. Much effort has been directed toward regenerating descending axons in an attempt to repair damaged connections; however, this has proven to be a very difficult challenge that does not guarantee the recovery of motor function. Recent studies have then highlighted the feasibility of targeting intraspinal mechanisms below the injury to improve locomotion in spinal animals (Jakeman et al, 1998; Jordan et al, 2014; Cowley et al, 2015). Although the spinal cholinergic system constitutes a very strong candidate for such approaches, surprisingly very little is known about its role in modulating locomotion in both intact and spinal transected preparations. The strength of this thesis rests in the findings that (1) intraspinal cholinergic

inputs have the ability to modulate motoneuron V_{th} and (2) this modulation is dependent on the physiological state of the spinal network. These observations are thus in line with an excitatory role of ACh in motoneuron excitability, probably serving to adjust motoneuron firing behaviour during motor output in spinal intact and acutely spinal transected animals, as argued in previous sections of this discussion. In the chronic spinal state, however, Jordan et al (2014) found evidence that the cholinergic system undergoes reconfigurations so that its effects turn to be deleterious for the production of locomotion. A few days following spinalization in adult cats and rats, ACh and/or EDRO induced sustained muscular contractions that interfered with the production of locomotion on a treadmill. This effect was attributed to a direct action of cholinergic agonists on motoneurons producing a tonic enhancement of the excitability of these neurons; however, direct evidence for this argument was not provided. Conversely, nicotinic and muscarinic receptor antagonists dramatically facilitated locomotion, even having a synergistic action with clonidine, a NA agonist that potently improves locomotion in spinal cats. These observations led Jordan et al (2014) to postulate that in the first few days after spinalization a "hyper-cholinergic state" of the spinal cord develops, interfering with the production of rhythmic activity. Somehow consistent with these findings, results in the present thesis demonstrated an excitatory effect, motoneuron V_{th} hyperpolarization, of ACh at low or moderate doses in quiescent networks or in association with the production of rhythmic activity; conversely, ACh produced an inhibitory action, V_{th} depolarization, in tonically active networks presumably associated with high levels of ACh and/or effects from other systems (see Paper 1, Figure 10). Whether the inability to appropriately activate motoneurons in order to generate locomotion in chronic spinal animals is due to either ACh-induced tonic V_{th} hyperpolarization (producing sustained muscle

activation) or ACh-induced V_{th} depolarization (increasing the amount of synaptic inputs required to recruit motoneurons) represents an interesting question to be investigated. However, indications that motoneuron V_{th} is depolarized following chronic spinal cord transections in adult cats (Hochman & McCrea, 1994) and rats (Beaumont et al, 2004) suggest that excessive ACh may trigger mechanisms to increase the V_{th} and limit motoneuron activation. Further, in order to elucidate the effects of the chronic "hypercholinergic state" on the overall excitability of spinal motoneurons, the interactions between ACh actions on V_{th} and other properties such as AHP and PICs in spinal intact and spinal transected animals need to be determined.

In addition to its effects on motoneurons, the undermining effects of ACh on locomotion following chronic spinalization were exerted through actions on the afferent pathways to spinal networks, which are a key component for the recovery of locomotion in spinal animals. Because nicotinic and muscarinic receptor antagonists powerfully increased the response amplitude of cutaneous reflexes in these animals, it was suggested that ACh inhibits afferent inputs important for CPG activation in the chronic spinal state (Jordan et al, 2014). It is likely that in addition to motoneurons and sensory pathways, other components of the locomotor CPG such as interneurons and neuromodulatory mechanisms, are also affected by the "hyper-cholinergic state". Only exhaustive analysis will determine how reconfigurations in the spinal circuitry and functionality following spinal cord injury affect the generation of movement. These reconfigurations, as the complex effects of ACh in functionally similar neurons observed in this thesis, are likely intricate and related to the specific characteristics of particular neurons and synapses. For example, changes in the number of cholinergic inputs to motoneurons following chronic spinal transections seem to

depend on the function of motoneurons, because lumbar flexor motoneurons show no change in C-bouton density, whereas both lumbar extensor and sacral motoneurons exhibit significant reductions in the number of these terminals after injury (Kitzman, 2006; Ichiyama et al, 2011; Skup et al, 2012; Kapitza et al, 2012). In sacral motoneurons, genes coding nicotinic receptor subunits as well as the VAChT transporter are up-regulated in response to spinal injury (Wienecke et al, 2010), probably contributing to the functional maladaptations of the cholinergic system in this state. In contrast to a detrimental role of ACh following injury, this modulator has been recently shown to stimulate the proliferation of ependymal cells with stem cell features located around the central canal in the spinal cord (Corns et al, 2015). This mechanism likely operates under injured conditions and thus may promote the restoration of spinal neuron function instead of hindering this process.

Together, Jordan et al (2014) and these studies demonstrate the impact of long-term adaptations in the intraspinal cholinergic system in relation to distinct components of the motor network, highlighting the role of this system in the modulation of movement. Findings in the present thesis contribute to the understanding of one important mechanism of such modulation, constituted by the regulation of motoneuron excitability through the V_{th} for action potential initiation. This understanding is essential for the design of therapeutic interventions aiming to facilitate functional recovery following spinal cord injury.

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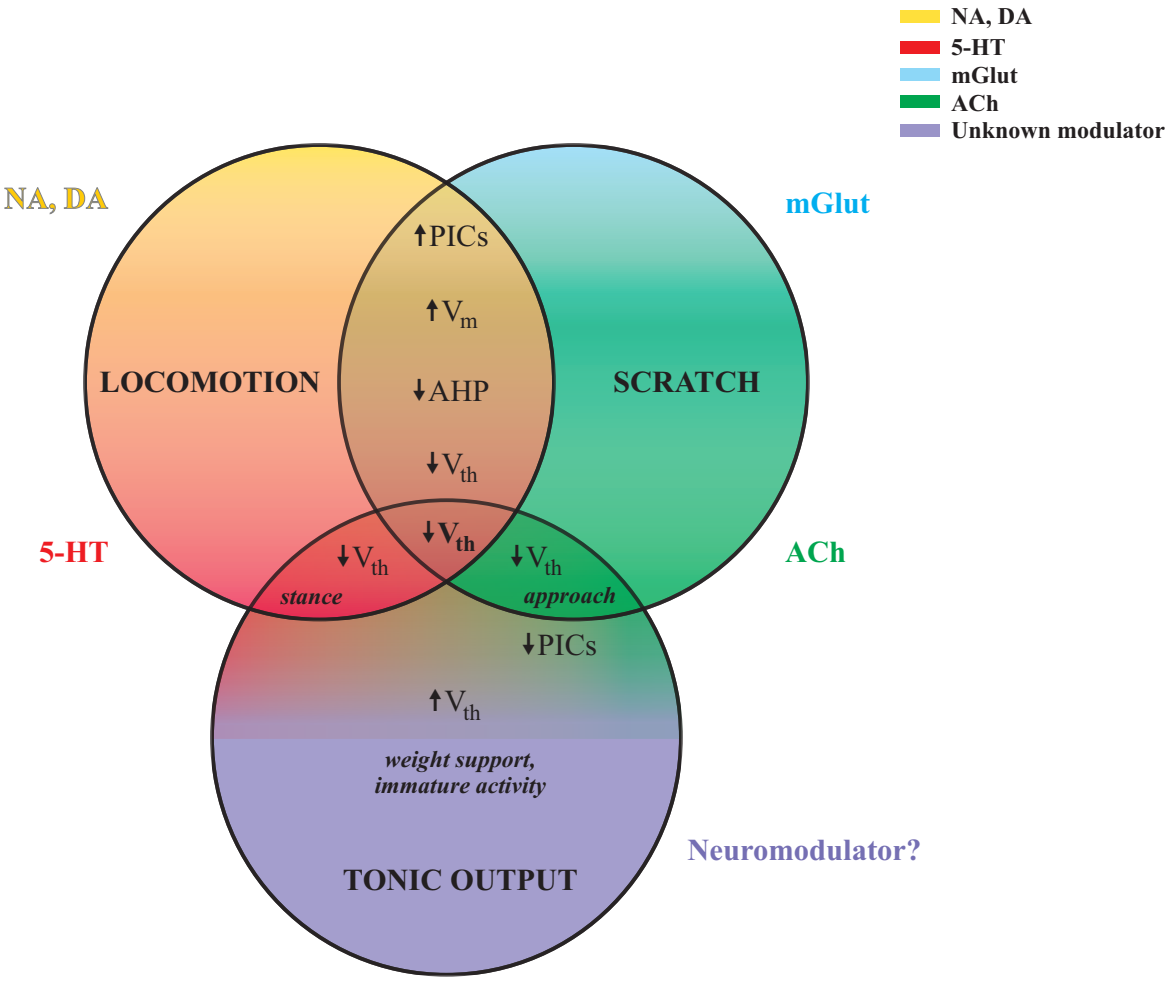
Figure I. Putative distribution of the neuromodulatory influences inducing state-dependent changes in distinct motoneuron electrical properties according to the motor behaviour produced by spinal networks.

Venn diagram conceptualizing the regulation of motoneuron excitability properties as a function of motor activity type and neuromodulatory systems activated. Circles represent different motor network outputs and colours represent distinct neuromodulatory systems activated during such motor output. The direction of change in motoneuron electrical properties is indicated by arrows as follows: ↑ increase, ↓ decrease. The position of each electrical property change in the diagram indicates its involvement during motor behaviour as well as the degree to which different neuromodulators are involved in mediating such change. See text for details.

Figure I


NA, DA
5-HT
mGlut
ACh
Unknown modulator
NA, DA
mGlut
↑PICs
↑Vm
LOCOMOTION
↓AHP
SCRATCH
↓Vth
↓Vth
5-HT
↓Vth
↓Vth
ACh
stance
approach
↓PICs
↑Vth
weight support,
immature activity
Neuromodulator?
TONIC OUTPUT