An Investigation of Voltage Threshold Hyperpolarization: Mechanism to Function

By

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A Thesis Submitted to the Faculty of Graduate Studies

In Partial Fulfilment of the Requirements for the Degree of

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Department of Physiology

Faculty of Medicine

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Abstract

Previous work has demonstrated that the onset of fictive locomotion induces a hyperpolarization of the voltage threshold (V_{th}) for action potential initiation. This powerful and consistent change in V_{th} revealed a novel mechanism used by the motor system to facilitate motoneuron firing during locomotor output. Given that V_{th} hyperpolarization is a relatively recent finding, there are many questions yet to be answered. The work comprising this thesis addresses V_{th} hyperpolarization from both mechanistic and functional perspectives.

Our first study (Paper 1) investigates the ionic mechanisms by which V_{th} hyperpolarization occurs through pharmacological modulation of voltage-gated sodium channels. In this study we made intracellular recordings from spinal motoneurons using an *in vitro* neonatal rat spinal cord preparation and human embryonic kidney (HEK) cells expressing the $Na_v1.2$ channel isoform. The results show that modulation of sodium channels induces V_{th} hyperpolarization through a negative shift in their voltage dependence of activation and does not require the enhancement of a persistent inward current.

Our second study (Paper 2) sought to determine whether V_{th} hyperpolarization occurs during motor behaviours other than locomotion and/or is dependent upon electrical brainstem stimulation. In this study we made intracellular recordings from spinal motoneurons in the adult decerebrate cat preparation and compared the V_{th} during quiescence and fictive scratch. The results show that V_{th} hyperpolarization occurs in the absence of electrical brainstem stimulation during fictive scratch, does not require motoneuron firing, and occurs following spinal transection.

We suggest that V_{th} hyperpolarization is a common mechanism used by the motor system to facilitate the recruitment and firing of spinal motoneurons during motor output, mediated by a change in the voltage dependency of sodium channels. Spinal motoneuron V_{th} hyperpolarization in the bigger picture of motoneuron excitability during motor output is discussed.

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Dedication

I dedicate this thesis to my family.

To my best friend and wife, Angela: the past five years have not always been easy for me, but I knew no matter what happened you would always be there to make me smile. You keep me grounded and your unconditional love and support is what kept me going when things got tough. You pushed me to succeed and without you I don't think I could have accomplished what I have. I hope I make you proud. I love you with every part of my being. Thank you.

To my son, Erik: never be afraid of a challenge. Work hard, never quit, and be confident that you can achieve anything you set your mind to.

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Finally to my parents: you provided me with confidence and encouragement to set my goals high and attain them. Thank you for your unending patience and support. After 25 years of school, I can finally say I know my alphabet!

List of Abbreviations

AHP	afterhyperpolarization
Ca^{2+}	calcium
CaP	persistent calcium
Cl^-	chloride
CPG	central pattern generator
ENG	electroneurogram
EPSP	excitatory post-synaptic potential
FF	fast fatigueable
FR	fatigue resistant
f/I	frequency / current
Hz	samples / second
I	current
I_{Cap}	persistent calcium current
I_{NaT}	transient sodium current
I_{Nap}	persistent sodium current
IS	initial segment
K^+	potassium
L-Dopa	L-3,4-dihydroxyphenylalanine
LDP	locomotor drive potential
MLR	mesencephalic locomotor region
mV	millivolt
NA	noradrenaline
nA	nanoampere
Na^+	sodium
NaP	persistent sodium
NaT	transient sodium
NM	non-medullated
PIC	persistent inward current
PKA	protein kinase A
PKC	protein kinase C
R	resistance
R_{in}	input resistance
S	slow
SD	soma-dendritic
SFA	spike frequency adaptation
SDP	scratch drive potential
TTX	tetrodotoxin
V	voltage
V_{th}	voltage threshold
5-HT	serotonin

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I. General Introduction

Alpha motoneurons (α-motoneurons) located within the spinal cord provide an essential link between the central nervous system and motor behaviour and were referred to as the "final common pathway" by Sherrington (1906) to emphasize the point that the nervous system must act directly or indirectly on motoneurons to produce movement, independent of its level of complexity (e.g. reflex or rhythmic). Motoneurons in the cat spinal cord were the first cells in the mammalian central nervous system recorded from using intracellular microelectrodes (Brock *et al.*, 1952). Since that time, a vast literature has accumulated describing their anatomy, physiology, and function, making them the most widely studied cells in the mammalian central nervous system.

Motoneurons were once seen as having a passive role in motor output. It was thought that motoneuron action potentials occurred by a simple summing together of excitatory and inhibitory synaptic input. It is now accepted that a complex process of neuronal integration, taking into account not only the passive properties of the motoneuron, but also its 'active' properties converts the synaptic drive to motoneurons arising from descending systems, sensory fibres, and the "central pattern generator" into action potentials during motor behaviour. Active properties play a vital role in the dynamic regulation of motoneuron excitability. Mechanisms capable of directly altering the response of motoneurons to their inputs, such as neuromodulators, thus have substantial effects on motoneuron recruitment, firing, and subsequent motor output.

In the General Introduction I will discuss information regarding motoneuron excitability, the majority of which focuses on a specific property of the motoneuron, the membrane potential at which an action potential is initiated: subsequently referred to as

the voltage threshold (V_{th}). This is followed by the two papers describing the work. It is concluded by a general discussion involving the potential implications of V_{th} hyperpolarization with respect to motor output.

Motoneuron Recruitment

The factors regulating the excitability and recruitment of spinal motoneurons have been an area of interest and controversy over the last half century. The process of motoneuron recruitment within a motor pool was first studied by Henneman and colleagues in the 1950's and 60's. In 1957, Henneman evoked reflexes in a spinalized cat by electrical stimulation of the dorsal roots. Simultaneous recordings of extracellular action potentials from ventral root filaments indicated that as stimulus strength increased, larger spikes began to fire. Based on the assumption that the recorded spike amplitude was a function of axon diameter, which was thought to be related to cell size, Henneman concluded by stating that the "susceptibility of neurons to discharge is a function of their size." These assumptions were later confirmed, namely that cell body size is positively correlated to with axon conduction velocity, which is in turn proportional to axon diameter (Cullheim, 1978). Henneman and colleagues went on to study variations in motoneuron excitability and muscle unit tension (Henneman *et al.*, 1965). They used the stretching of hindlimb muscles (soleus and gastrocnemius) to activate motoneurons via muscle spindle activation (i.e. stretch reflex) to study the size of ventral root action potentials in decerebrate cats. They observed that small extracellular action potentials were recruited prior to those with larger action potential sizes. It was concluded that the size of the cell determines its threshold for action potential initiation. This conclusion led to the coining of the term "size principle."

The size principle is logical according to Ohm's law (I=V/R) in that when given a fixed synaptic current, the motoneuron input resistance (R_{in}) will determine the change in somatic membrane potential. R_{in} measured in cat motoneurons *in vivo* exhibit about a 10 fold range (0.4 to 4.0 MOhms) (Gustafsson & Pinter, 1984). In accordance with the size principle, average R_{in} values are largest in slow (S) motoneurons, compared to fast fatigue resistant (FR) and fast fatigueable (FF) motoneurons (Fleshman *et al.*, 1981) due to a smaller surface area results in fewer ion channels in parallel. Thus, smaller motoneurons, presumably type S, would require less synaptic current to depolarize to threshold and be recruited to fire action potentials.

It has become evident (Gustafsson & Pinter, 1984) that size alone cannot fully explain the recruitment of spinal motoneurons. Current evidence suggests that the recruitment of spinal motoneurons depends on a combination of intrinsic (e.g. cell size, time constant, and specific membrane resistivity) and extrinsic (e.g. synaptic input) factors. One such factor is the V_{th}.

The Voltage Threshold

Voltage Threshold Defined

The excitability of motoneurons is determined by their passive and active properties and is modulated via pre-motoneuronal networks. A single motoneuron may be innervated by approximately 20,000 presynaptic endings, any number of which may provide excitatory or inhibitory input (Ornung *et al.*, 1998). These competing effects are integrated by the motoneuron to determine whether an action potential will be initiated. In general, an action potential occurs when the membrane potential is depolarized

sufficiently to reach threshold. Whether threshold is reached depends on the sum of the synaptic current reaching the action potential trigger zone, the axon hillock, also known as the initial segment (IS).

An action potential is initiated when the postsynaptic response is of sufficiently depolarized amplitude, and fast enough to avoid sodium (Na^+) channel inactivation due to accommodation. Most spinal excitatory postsynaptic potentials (EPSPs) are believed to result from the opening of both Na^+ and potassium (K^+) channels. The widely accepted understanding of action potential initiation is that an action potential occurs when inward Na^+ current overwhelm evoked and resting K^+ outward currents. Since Na^+ channels are activated by membrane depolarization, raising the membrane potential just above the balance point for Na^+ and K^+ currents results in a rapid increase in Na^+ channel opening and hence action potential initiation. If the EPSP is small, the amount of K^+ that exits the cell via the 'leak channels' is large enough to restore resting membrane potential and no action potential is initiated. If the EPSP is large, K^+ current exiting the motoneuron through the leak channels is not capable of preventing the activation of voltage-gated Na^+ channels. The result is a rapid influx of Na^+ which causes depolarization and activation of adjacent Na^+ channels driven by the Na^+ concentration and voltage gradients (Hodgkin & Huxley, 1952a). This system acts as a positive feedback loop to further depolarize the cell with the membrane potential coming close to the Na^+ equilibrium potential (Coombs *et al.*, 1957). Thus, V_{th} represents the point at which the inward Na^+ current overcomes the outward K^+ current and an action potential is initiated. Modulation of V_{th} will have direct effects on the excitability of the motoneuron, either facilitating (V_{th} hyperpolarized) or inhibiting (V_{th} depolarized) motoneuron recruitment and/or firing.

Voltage Threshold and Recruitment

In anesthetized cats investigations of motoneuron properties using V_{th} as a reference for recruitment have failed to show a consistent or strong relation between V_{th} and recruitment order. Pinter el al. (1983) investigated V_{th} in the cat and concluded no significant differences existed between the V_{th} of slow and fast motoneurons elicited via monosynaptic EPSPs. Additionally, they determined that V_{th} did not covary with R_{in} (typically larger for slow motoneurons), afterhyperpolarization (AHP) duration, or motor unit twitch contraction time. They instead suggested that V_{th} would be a function of the balance of excitatory/inhibitory connections or density of synaptic input to the motoneuron. Similarly, Gustafsson and Pinter (1984) studied motoneuron properties and their relation in determining intrinsic excitability within a motoneuron pool. They grouped cells according to their AHP duration or rheobase current, producing groups of motoneurons with properties similar but not identical to the motor unit types generally discussed in the literature (i.e. S, FR, FF). They found that there was a "tendency" for V_{th} to be lowest in motoneurons with low rheobase and high R_{in}, presumably type S. V_{th} can also have large variations within motoneuron pools. For example, as can be seen in Table 1 of Krawitz and colleagues (2001), the V_{th} of 10 antidromically identified medial gastrocnemius motoneurons was reported. Their data indicate that although the motoneuron species was the same, the V_{th} was quite variable (range: -64.4 mV to -35.2 mV) as was rheobase current (range: 2.0 nA to 26.4 nA) in the quiescent state. Thus it is apparent that V_{th} variations exist between and within motoneuron species.

Voltage Threshold Depolarization

The V_{th} for action potential initiation is not a fixed value. Kolmodin and Sköglund (1958) studied repetitive firing during synaptic excitation. They showed that firing levels in different motoneurons was not the same and also that during repetitive firing, the V_{th} of early spikes in the train was most often more hyperpolarized than those from later spikes (e.g. between -55 and -48 mV, initial and final V_{th} in a train, respectively). The tendency for V_{th} to depolarize with successive action potentials is known as accommodation, and highlights another factor that influences motoneuron recruitment - the speed at which the depolarization occurs.

If depolarization is slow, an action potential may not be elicited because of the voltage-dependent inactivation of the Na^+ conductance (i.e. Na^+ channels will become activated but action potential initiation may be delayed because the Na^+ channels will also have time to inactivate). Transition from this inactivated state requires hyperpolarization. If there is a slow, continuous depolarization, this counters the hyperpolarization required to reset the Na^+ channels, making them unavailable for further activation. In addition, an efflux of K^+ through K^+ channels with depolarization will bring the membrane potential back toward the resting membrane potential (i.e. more negative). Thus, during slowly rising or sustained depolarizations the combined actions of Na^+ channel inactivation and K^+ channel activation leads to a depolarization of V_{th} through a reduction in the inward Na^+ currents and enhancement of the outward K^+ currents.

Depolarization of V_{th} has also been demonstrated physiologically as a means of training induced spinal plasticity. Carp and Wolpaw (1994) used operant conditioning in monkeys to demonstrate motoneuron plasticity by a reduction in the amplitude of the H-

reflex. Intracellular recordings of the trained muscle group (triceps surae) demonstrated a more depolarized V_{th} than the non-trained limb. Depolarization of V_{th} has also been shown following decreased motoneuron activity as a result of hindlimb unweighting of adult rats (Cormery *et al.*, 2005) and spinalization of adult cats (Hochman & McCrea, 1994) and rats (Beaumont *et al.*, 2004). Cormery et al. (2005) showed that 2 weeks of hindlimb unweighting decreased motoneuron excitability as evident by decreased spike amplitude, increased rheobase current, and a 5 mV depolarization of V_{th}. Following 6 weeks of spinalization, Hochman and McCrea (1994) demonstrated a depolarization of V_{th} in adult cats similar to the findings in adult rat motoneurons following 4 weeks of spinalization (Beaumont *et al.*, 2004). Interestingly enough, motoneuron V_{th} depolarization following spinalization in rats was preventable by the application of a passive cycling exercise of the hindlimbs for 1 hour/day, 5 days/week, implying that exercise may counter motoneuronal adaptations seen following decreased activity (i.e. hindlimb unweighting and/or spinalization). Together these studies demonstrate that decreases in neuromuscular activity can lead to V_{th} depolarization, that would contribute to a decrease in motoneuron excitability. Conversely, increases in neuromuscular activity or simply performing a motor output may be accompanied by V_{th} hyperpolarization and thus an increase in motoneuron excitability.

Voltage Threshold Hyperpolarization

Motoneuron adaptations following long-term sensitization training of the siphon withdrawal reflex in *Aplysia* included a hyperpolarization of V_{th} (Cleary *et al.*, 1998) while Beaumont and Gardiner (2003) showed that 16 weeks of endurance treadmill training (i.e. 2h/day) induced V_{th} hyperpolarization in adult rat motoneurons. These

experiments compared V_{th} in motoneuron populations before and after the intervention. The lowering of the mean V_{th} in these preparations indicates the ability of motoneurons to undergo chronic adaptations as a result of increased activity levels.

Krawitz et al. (2001) provided the first description of a V_{th} change where each cell served as its own control. This study also provided the first description of a state-dependent V_{th} hyperpolarization using brainstem-evoked fictive locomotion in the decerebrate cat. V_{th} hyperpolarization occurred at the onset of fictive locomotion and recovered within seconds following the termination of locomotor activity. This effect on V_{th} was immediate, robust (mean change of -8.0 mV), and occurred in every motoneuron examined including flexors and extensors. The amount of V_{th} hyperpolarization was independent of rheobase values, suggesting that it occurred in both slow and fast motoneurons. Furthermore, V_{th} hyperpolarization occurred during both the depolarized and hyperpolarized phases of the locomotor step cycle. Thus it was concluded that V_{th} did not depend on the rhythmic depolarization per se and did not rely on rhythmic firing. It is also important to note that V_{th} hyperpolarization was not associated with obvious changes in action potential amplitude or shape. It was further suggested that because V_{th} hyperpolarization persisted for a few seconds following a bout of fictive locomotion, that a neuromodulatory mechanism may have mediated the effect.

Since this rapid state-dependent lowering of V_{th} was a newly recognized feature of central nervous system motor systems, the underlying mechanisms were not known to Krawitz et al. (2001) and remain incompletely understood to date. An attempt was made to explore potential mechanisms for V_{th} hyperpolarization using a computational model of the spinal motoneuron in which a variety of conductances could be changed to alter V_{th}

in a manner similar to that occurring during fictive locomotion (Dai *et al.,* 2002). Dai et al. (2002) used triangular injected current ramps with superimposed square pulses to measure V_{th} under control conditions and following the modulation of Na^+ and K^+ conductances. They concluded that although cyclic membrane depolarization could induce some V_{th} hyperpolarization, the effects were relatively small and less effective than modulating Na^+ and K^+ conductances. This study is discussed further below.

Because V_{th} remained hyperpolarized in the period immediately following the cessation of fictive locomotion, Krawitz et al. (2001) posited that V_{th} hyperpolarization was mediated via a neuromodulatory pathway. Presumably the activation of some (unknown) modulatory system at the onset of fictive locomotion induced the post synaptic changes in motoneurons leading to V_{th} hyperpolarization. Obvious candidates were the monoamine neurotransmitters released from descending projections originating within the brainstem. Motoneurons in the spinal cord are innervated densely by monoaminergic input (serotonin: 5-HT; noradrenaline: NA). 5-HT is a monoamine of predominantly supraspinal origin that has been shown to increase the excitability of motoneurons. The anatomical correlates containing 5-HT cells include the raphe pallidus, raphe obscures, and the raphe magnus, all of which are located in the brainstem and project to the cervical and lumbar cord (Dahlstrom & Fuxe, 1965). NA containing cells within the locus coeruleus (Gilman, 1992) provide an additional descending monoaminergic pathway shown to increase motoneuron excitability (Fedirchuk & Dai, 2004). Given that the discharge frequency of the 5-HT containing cells within the brainstem increases during repetitive types of motor output such as locomotion (Jacobs & Fornal, 1993), and that 5-HT exerts tonic facilitatory effects on spinal hindlimb

motoneurons, it is possible that during midbrain (MLR) stimulation lower brainstem pathways were also activated and monoamines released (Jordan *et al.*, 2008), thus enhancing motoneuron excitability.

Research on an isolated brainstem spinal cord neonatal rat preparation has made it possible to explore monoaminergic mechanisms as potential mediators of V_{th} hyperpolarization. Gilmore and Fedirchuk (2004) used an *in vitro* brainstem and spinal cord attached preparation from neonatal rats to investigate whether V_{th} hyperpolarization was mediated by an endogenous, descending, monoaminergic pathway and whether or not V_{th} hyperpolarization was locomotor dependent. They demonstrated that cooling of the cervical cord blocked V_{th} hyperpolarization and ventral root activity during electrical brainstem stimulation, as did the addition of a 5-HT_{2A} receptor antagonist, ketanserin. These results implicated the serotonergic descending pathway as one mechanism potentially responsible for the V_{th} hyperpolarization during fictive locomotion. The ability of the monoamines to induce V_{th} hyperpolarization was confirmed by Fedirchuk and Dai (2004) who showed that bath application of either 5-HT or NA could induce V_{th} hyperpolarization in ventral horn neurons of an isolated neonatal rat spinal cord preparation.

While monoamines can modulate V_{th}, the question of which channel(s)/conductance(s) are modulated to produce state-dependent changes in V_{th} remains unanswered.

Mechanisms of Voltage Threshold Hyperpolarization

Active Conductances

In addition to the conductances responsible for the action potential, namely Na^+ and K^+, motoneurons possess an assortment of time and voltage-dependent ion channels that alter motoneuron excitability. The voltage-dependent ion channels that have major influences on the excitability of spinal motoneurons include, but are not limited to (Rekling *et al.,* 2000): (**1**) transient Na^+ (NaT) channels responsible for the rising phase of the action potential, (**2**) persistent Na^+ (NaP) channels that activate below spike threshold to amplify synaptic input, (**3**) Ca^{2+} dependent K^+ channels responsible for the post-spike AHP and thus regulate firing frequency, (**4**) leak channels (predominantly K^+) which stabilize membrane potential, and (**5**) persistent Ca^{2+} (CaP) channels which facilitate non-linear integration of synaptic inputs and plateau potentials underlying sustained firing and bistability. Others include: (**1**) various K^+ channels that contribute to repolarization and thus help shape action potential trajectory, such as: the delayed rectifier (K_{DR}), transient outward (I_A), Ca^{2+} activated K^+ channels [$I_{KCa(BK)}$ and $I_{KCa(SK)}$]; and (**2**) inward rectifier channels (K_{ir} and I_h) that reduce conductances during depolarization and increase with hyperpolarization. Many of these voltage-gated channels are in turn under the influence of modulatory actions, thereby affecting the excitability of the motoneuron. So which, if any, of these channels is mediating V_{th} hyperpolarization during fictive locomotion?

Putative Ionic Mechanisms of V_{th} hyperpolarization

The ionic mechanism(s) and channel(s) responsible for V_{th} hyperpolarization are

unknown; however putative mechanisms have been proposed. As discussed by Gilmore and Fedirchuk (2004), 5-HT has been shown to depolarize neurons by increasing a slow inward current carried by Na^+ and K^+, facilitation of a low voltage-activated Ca^{2+} current, and inhibition of a fast inward K^+ current. Dai et al. (2002) investigated the potential ionic mechanisms of V_{th} hyperpolarization using models of motoneurons innervating S, FR, and FF muscle fibres with properties resembling those of real motoneurons *in vivo*. Ten active motoneuron conductances were included in the motoneuron model: fast sodium (g_{Na}), persistent sodium (g_{NaP}), delayed rectifier potassium ($g_{K(DR)}$), A-current ($g_{K(A)}$), calcium dependent potassium ($g_{K(AHP)}$), T-type calcium (g_{CaT}), L-type calcium (g_{CaL}), N-type calcium (g_{CaN}), h-current (g_h), and potassium leak current (g_{leak}). The maximal conductance and/or their voltage dependency of activation and inactivation was altered for each channel type to try and emulate the V_{th} hyperpolarization seen during fictive locomotion in the cat. The modulation of two main conductances was found to induce V_{th} hyperpolarization similar to that seen during fictive locomotion in the cat without inducing significant changes in action potential shape: (1) increasing initial segment gNa by 50%; (2) hyperpolarization of the initial segment voltage dependency for gNa; (3) 70% reduction in initial segment gK(DR); (4) depolarization of the initial segment voltage dependency for gK(DR). All four changes could induce V_{th} hyperpolarization by facilitating the net inward current at substhreshold membrane potentials. They also concluded that enhancing the gNaP was an unlikely candidate for V_{th} hyperpolarization since its modulation resulted in large effects on action potential shape with only a modest hyperpolarization of the V_{th}.

The conclusions from the modeling, namely that modulation of the transient Na^+

current (I_{NaT}) generating the action potential could induce V_{th} hyperpolarization and that the persistent Na^+ current (I_{NaP}) was an unlikely contributor, provided the basis for the research presented as Paper 1 in this thesis. Thus, given the potential role of Na^+ channel mediated V_{th} hyperpolarization, the following sections will focus on the voltage-gated Na^+ channels structure and function.

Voltage-gated Sodium Channels

The Initial Segment

Early intracellular recordings from motoneurons showed that the rising phase of the action potential consisted of two components. The components consisted of a 'small' and 'large' spike, referred to as the non-medullated (NM) and soma-dendritic (SD) spikes, respectively (Brock *et al.*, 1953). The NM spike is now commonly referred to as the IS spike and it is believed to be the site for action potential initiation (Brock *et al.*, 1953). Coombs et al. (1957) demonstrated that the IS spike could be elicited in the absence of the SD spike by hyperpolarizing the soma, with the mean threshold depolarizations for generating IS and SD spikes being 10 and 30 mV, respectively. The lower V_{th} for activation of IS spikes can partially be attributed to differences between the density of Na^+ channels within each region of the cell. For example, in cultured spinal neurons the density of Na^+ channels is 7 times higher on the IS than on the soma (Catterall, 1981). As the rising phase of action potential is due to a rapid influx of Na^+ ions, it is reasonable that the area of the motoneuron containing the highest density of voltage-gated Na^+ channels would be the site of action potential initiation. A greater density of Na^+ channels would effectively reduce the amount of depolarization required

to reach V_{th} by reducing the percentage of Na^+ channels that must be activated. In other words, at any given level of depolarization, a larger number of Na^+ channels would be activated, inducing an action potential at more hyperpolarized membrane potentials. Action potentials generated at the IS would provide the added benefit of giving a common site of synaptic integration from input arising from the dendrites and soma. According to this idea, the SD spike results from back propagation of the IS spike.

Structure and Neuromodulation of Voltage-Gated Sodium Channels

Mammalian voltage-gated Na^+ channels contain one large α-subunit (~250 kDa) and one or two smaller ß-subunits (~30-40 kDa; ß1, ß2. ß3, and/or ß4) that modifies channel function (Isom *et al.*, 1992). The α-subunit forms the functional voltage-gated Na^+ channel and consists of four homologous repeat domains (D1 to D4) each with six transmembrane segments (S1 to S6) (Catterall, 2000). The voltage sensor is located in the positively charged S4 segments which may move during depolarization and modify an "activation gate" on the S6 segment. The inactivation gate is thought to reside at the cytoplasmic linker between D3 and D4 (Hille, 2001). Generation of the action potential by Na^+ channels represents the final neural output of the motoneuron. It is becoming increasingly evident that voltage-gated Na^+ channels are a target for neuromodulation in a variety of cell types.

Modulation of Na^+ channels has been demonstrated to be produced by dopamine and acetylcholine through activation of protein kinase A (PKA) (Cantrell *et al.*, 1997) and protein kinase C (PKC) (Cantrell *et al.*, 1996) pathways, respectively. In neostriatal cells, dopaminergic modulation (by a PKA pathway) of Na^+ channels has been demonstrated with varying effects depending on the receptor type activated. For example,

D1 receptor activation inhibits peak Na^+ current and depolarizes V_{th} for action potential initiation while activation of D2 receptors enhances the peak Na^+ current and accelerates depolarization (Surmeier and Kitai, 1997). Na^+ channel modulation has also been suggested to contribute to slowing of firing rates in pyramidal cells through depolarization of V_{th} (for review see Cantrell & Catterall, 2001). Varying effects based on activation of the PKC pathway have also been reported. In rat hippocampal neurons, activation of muscarinic receptors reduced peak Na^+ current without affecting activation/inactivation kinetics of Na^+ channels (Cantrell *et al.*, 1996). In contrast, Franceschetti et al. (2000) demonstrated that activation of the PKC pathway produced a hyperpolarizing shift in NaT channel activation (excitatory) and steady-state inactivation (inhibitory), as well as a decrease in I_{NaT} peak amplitude. PKC pathway activation has also been shown to induce V_{th} hyperpolarization (Astman *et al.*, 1998).

Using whole cell patch clamp recordings in ventral horn neurons in slice from neonatal rats, Dai et al. (2009) investigated whether 5-HT induced V_{th} hyperpolarization was mediated via a PKC dependent pathway. Contrary to his hypothesis PKC pathway activation decreased excitability by reducing persistent inward current (PIC) amplitude, depolarizing PIC onset, and a decrease in I_{NaT} amplitude. PKC activation had variable effects on V_{th}, causing either no change or a depolarization. 5-HT however induced a V_{th} hyperpolarization and was not altered by PKC activation or inactivation, suggesting the modulation of V_{th} could be mediated via multiple pathways (Dai *et al.*, 2009).

Regardless of our scanty knowledge of the pathway(s)/mechanism responsible for Na^+ channel modulation, voltage-gated Na^+ channels are clearly a site for potential neuromodulation. Neuromodulators such as 5-HT and NA may thus induce V_{th}

hyperpolarization by modulating voltage-gated Na^+ channels during locomotion.

Persistent Inward Currents

As previously discussed there are two main types of currents generated by Na^+ channels (transient and persistent). To date however, it is not known whether the same or different channel(s) generate the I_{NaT} and the I_{NaP}. The contribution of I_{NaP} to the total PIC and its importance to motoneuron excitability however has been demonstrated. The following sections will discuss PICs and motoneuron excitability, with a focus on the I_{NaP}.

Dendrites and Motoneuron Excitability

Although the soma and axon hillock of α-motoneurons are covered by synaptic boutons, the dendritic tree constitutes approximately 97% of the total membrane surface area (Rekling *et al.*, 2000), and thus have the majority of synaptic contacts. The number of synaptic boutons has been estimated to be between 50,000 and 140,000, originating from approximately 20,000 synapses (Ornung *et al.*, 1998). Thus, the dendrites play a significant role in the response of motoneurons as current passes from the dendrites to the axon hillock, where synaptic integration and action potential initiation occur (Safronov *et al.*, 1997). The dendrites, once believed to be passive, also contain voltage-dependent, non-inactivating channels that generate PICs and thus influence motoneuron excitability. The dendritic origin of PICs however is based on indirect evidence as it has not yet been possible to record from the dendrites of spinal motoneurons (for review of PIC origin see Heckman, 2003).

Persistent Inward Current Terminology

A PIC is a voltage-gated inward current that is activated at subthreshold membrane potentials and enhances cell excitability. PICs can be visualized using intracellular voltage-clamp recordings and are described as a region of "negative slope conductance" in the current-voltage relationship (Schwindt and Crill, 1977). The term "negative slope conductance" arises from recordings whereby during a linearly rising voltage ramp, there is a strong downward deflection in the net current. If the PIC is large relative to the leak conductance, it induces a region of negative slope conductance in the net current-voltage relationship. An important characteristic of PICs is that the deactivation of PIC on the descending limb of a voltage command occurs at a more hyperpolarized membrane potential than does PIC activation on the ascending limb, a process known as hysteresis (Heckman *et al.,* 2008).

PICs can also be visualized as plateau potentials, self-sustained firing, and bistability during intracellular current-clamp recordings (Heckman, 2003). When the cell is held at a hyperpolarized level, a brief period of excitatory synaptic input generates an equally brief period of cell depolarization. Following inactivation of Na^+ spikes by tetrodotoxin (TTX), if the same excitatory input is given to the cell while at a more depolarized membrane potential close to the threshold for action potential initiation, there is a marked depolarization or 'jump' in membrane potential that remains following removal of the excitatory input. This is referred to as a plateau potential. If the same input is given without the blockade of Na^+ spikes, a PIC may be expressed as a marked acceleration in the firing frequency of the cell in response to a constant excitatory input. After the excitatory input has been removed, the ability of the cell to maintain the ability

to fire is referred to as self-sustained firing. A subsequent brief hyperpolarization or inhibition arising from a different synaptic input can end cell firing and return the cell to the quiescent state (Heckman, 2003) The ability of the motoneuron to possess two stable states of activity, one around resting membrane potential and the other at a more depolarized potential is referred to as bistability.

Persistent Inward Currents and Spinal Motoneurons

Evidence for PICs in spinal motoneurons was first described in a series of experiments by Schwindt and Crill (1977, 1980a, and 1980b) in the cat. They characterized the PIC by reducing outward currents using penicillin (Schwindt & Crill, 1980a) or tetraethylammonium (Schwindt & Crill, 1980b) to block Cl^- and K^+ currents, respectively, and thus 'uncovering' the PIC, that they determined to be a voltage-dependent Ca^{2+} current. Around the same time, Hultborn and coworkers (1975) demonstrated that a single volley in group Ia afferents could produce prolonged discharges in motoneurons lasting for several seconds. They postulated the existence of a "reverberating loop" with prolonged discharges in interneurons causing prolonged discharges of motoneurons. This finding prompted the subsequent studies on plateau potentials by Hultborn and colleagues.

Studying spinal motoneurons in the cat, Hultborn and colleagues (Crone *et al.*, 1988; Hounsgaard *et al.*, 1988) used brief excitatory synaptic inputs to the spinal cord and demonstrated a prolonged muscle contraction involving the serotonergic system, although the idea that this was due to a PIC was not formulated at that time. The existence of a PIC capable of inducing a depolarized state in mammalian α-motoneurons

and its possible implications for motor output dramatically changed the landscape for motoneuron research. Indeed, research conducted on PICs in motoneurons and their possible implications in motor control and in such diseases as spasticity and amyotropic lateral sclerosis has grown substantially.

The picture put forth by Schwindt and Crill regarding PICs and plateau potentials in the late 70's and early 80's however was incomplete. Since these initial experiments, much has been learned about PICs and their role in motoneuron excitability. A series of *in vivo* cat experiments by Hultborn and colleagues (Hounsgaard *et al.*, 1984; Conway *et al.*, 1988; Crone *et al.*, 1988; Hounsgaard *et al.*, 1988) demonstrated bistability in α-motoneurons in the decerebrate cat in response to synaptic input or brief depolarizing pulses. Bistability however was eliminated following spinal transection and recovered via 5-HT or L-DOPA administration. Because bistability was dependent upon depolarization of the motoneuron in the presence of monoamines, Crone and colleagues (1988) suggested that maintained cell excitability was dependent upon active descending serotonergic and noradrenergic inputs which were hypothesized to be tonically active in the decerebrate preparation (Crone *et al.*, 1988; Conway *et al.*, 1988; Hounsgaard *et al.*, 1988). The importance of monoaminergic input in regulating motoneuron excitability was further demonstrated by Lee and Heckman (2000). They investigated the ability of different levels of monoaminergic input to amplify synaptic input to spinal motoneurons. They demonstrated that in a "minimal neuromodulatory state," defined as a a pentobarbital-anesthetized preparation in which PIC is small, activation of the Ia afferents by a high-frequency, low amplitude sinusoidal vibration (180 Hz; 80 μM peak-to-peak) of the medial and lateral gastrocnemius nerves generated about 3 nA at the soma.

However in the presence of "moderate monoaminergic" input (decerebrate preparation) the synaptic current was increased about fourfold. Further activation of monoamine receptors by exogenous topical application of methoxamine to the ventral surface of the spinal cord enhanced the synaptic current sixfold. These findings are in line with those of Hultborn and colleagues and further illustrate the importance of monoamines to motoneuron excitability. In addition, Heckman and Lee (2000) demonstrated that spinal transection eliminated a voltage-dependent amplification of the peak inward current as was the case in the studies by Hultborn and colleagues (Crone *et al.*, 1988; Conway *et al.*, 1988; Hounsgaard *et al.*, 1988). A recent and important finding is that PICs are present in motoneurons in ketamine-xylazine anaesthetized adult rats, providing an alternative to decerebrate and pentobarbital-anesthetized preparations for the study of PICs (Button et al., 2006).

In vitro turtle spinal cord experiments (Hounsgaard & Kiehn, 1985; Hounsgaard & Kiehn, 1989) were subsequently used to describe the dependence of PIC expression on the monoamines as well as its cellular mechanisms. Specifically, it was possible to demonstrate that an L-type Ca^{2+} current was essential for 5-HT induced bistable behaviour likely through suppression of a K^+ current. It is now recognized that PICs are generated by non-inactivating or slowing inactivating voltage-gated channels activated just above the resting membrane potential (Lee & Heckman, 1998). In addition to the Ca^{2+} current investigated by Hounsgaard and Kiehn (1989), the importance of a TTX-sensitive, I_{NaP} underlying the PIC in motoneurons has also been established. The relative contributions of each of the Ca^{2+} and Na^+ currents in generating the PIC has been estimated at approximately 60% and 40%, respectively in adult rat sacral motoneurons

(Harvey *et al.*, 2006). The role of I_{NaP} in regulating motoneuron excitability has gained much attention in recent years and will thus be the focus of the following sections.

Origin of the Persistent Na^+ Current

The origin of the persistent Ca^{2+} current (I_{CaP}) has been attributed to L-type Ca^{2+} channels in both turtle (Hounsgaard & Kiehn, 1989) and mouse (Carlin *et al.*, 2000) spinal motoneurons. L-type Ca^{2+} channels are blocked by nifedipine and are but one source of Ca^{2+} currents in neurons (Catterall et al., 2005b). The existence of distinct Na^+ channel types for the rapidly inactivating and persistent Na^+ currents has yet to be settled. Three main theories as to the origin of the I_{NaP} have been proposed: (1) a "window current" based on the Hodgkin-Huxley model of whole cell current properties (Hodgkin & Huxley, 1952b); (2) a subtype of NaT channels that does not inactivate (French *et al.*, 1990; Brown *et al.*, 1994); (3) a change in the inactivation properties of the NaT channels (Alzheimer *et al.*, 1993). For review see Crill (1996).

The window current is based on the Hodgkin-Huxley model (Hodgkin & Huxley, 1952b). The Hodgkin-Huxley description of action potential initiation and shape was based upon their hypothesis that voltage-gated channels could be regulated by separate factors controlling activation and inactivation. Hodgkin and Huxley developed equations tht described the temporal characteristics of these two processes (Hille, 2001). According to the window current hypothesis of prolonged Na^+ channel activation there exists a small voltage range whereby the probability of a Na^+ channel not being inactivated (i.e. available for activation) and the probability of a Na^+ channel being activated are both greater than zero, thus producing a steady or persistent Na^+ conductance. However this seems unlikely based on experimental findings. French et al. (1990) calculated the Na^+

window conductance and compared it with I_{NaP} in pyramidal cells. They showed that calculated window conductance increased rapidly with depolarization and then decreased with further depolarization eventually falling to zero. In contrast, the recorded I_{NaP} increased monotonically and did not decrease. They suggested that the I_{NaP} may be generated by Na^+ channels closely resembling those of the NaT but with a different voltage dependency. The possibility that different Na^+ channels mediate I_{NaP} and I_{NaT} is supported by the finding that the activation of I_{NaP} occurs at ~10 mV negative to the I_{NaT} (Brown *et al.*, 1994; French *et al.*, 1990). A different channel subset however is not the only possible explanation.

Another suggestion for the Na^+ channels mediating I_{NaP} is that they differ from those generating I_{NaT} by differences in their activation states or modes. For example, Brown et al. (1994) found that the activation curve for I_{NaP} was about 7 mV negative to that of I_{NaT} in pyramidal neurons. When papain was added to the cells to remove the inactivation gate on channels generating the I_{NaT}, its activation curve shifted to more hyperpolarized levels and was activated at the same membrane potentials as the I_{NaP}. They concluded that the differences between the voltage dependence of activation between persistent and transient Na^+ currents were insufficient to suggest generation via different Na^+ channels. They suggested that the I_{NaP} arises from a subset of NaT channels that lose their ability to inactivate.

Generation of a I_{NaP} has also been attributed to gating changes in NaT channels. Alzheimer et al. (1993) studied the kinetics of Na^+ channel gating in rat and cat sensorimtor cortex using either slices or acutely isolated neurons. Following 400 ms depolarizing voltage steps, Na^+ channels generated different types of channel openings,

referred to as brief late openings, sustained openings, and early openings. Based on the almost identical amplitude and slope conductance of each of the Na^+ channel opening modes, they concluded that a uniform population of Na^+ channels were responsible for the early and recurrent openings. Taddese and Bean (2002) investigated the origin of the I_{NaP} that drives pacemaking in tuberomammillary neurons. They found that when the Na^+ channels generating the I_{NaT} were driven into inactivation by high-frequency stimulation, the I_{NaP} decreased in parallel. This led them to suggest that the I_{NaP} originated from subthreshold gating of the same Na^+ channels generating the I_{NaT}.

Characterization of the Na^+ channels that generate the I_{NaP} in spinal motoneurons is an important issue in the study of motor output. If in fact the I_{NaP} were carried by a separate channel, tools could be developed for the separation and/or isolation of these currents. This would allow investigation on the effects of modulating each current on functional motoneuron properties and motor output. Some insights into the Na^+ channel(s) generating I_{NaP} can be gathered from results in pyramidal cells and spinal motoneurons. In these studies functional differences have been inferred among the Na^+ channels from correlations between the levels of Na^+ channel isoform expression and electrophysiological characteristics.

A variety of different Na^+ channels have been identified. To eliminate confusion resulting from multiple names given to the same channels, a standardized nomenclature has been developed (Goldin *et al.,* 2000). A numerical system is used to define subfamilies and subtypes based on similarities between the amino acid sequences of the channels. In this nomenclature system, the name of a specific channel is made up of the main permeating ion (Na^+) followed by a subscript indicating its' main physiological

regulator (voltage). The number following the subscript represents the gene subfamily (Na_v1), and the number following the decimal identifies the specific channel isoform (e.g. $Na_v1.1$) (Catterall *et al.,* 2005a). Within the central nervous system, $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$ are expressed to varying degrees (Goldin, 2001) around the time of birth, with all but the $Na_v1.3$ being highly expressed in adult rats (Beckh *et al.*, 1989). There are however, developmental shifts in the Na^+ channel expression levels during the early postnatal period. At birth the ventral horn of rat spinal cords express the Na^+ channel subtype mRNA for $Na_v1.1$, $Na_v1.2$, and $Na_v1.3$ (Felts *et al.*, 1997). The expression of $Na_v1.6$ mRNA becomes detectable at the second postnatal day (P2) and increases until the third postnatal week. This finding is in general agreement with electrophysiological data collected from mutant mice lacking the Scn8a gene encoding the $Na_v1.6$ channel (Garcia *et al.*, 1998). Garcia et al. (1998) showed that wild-type mice demonstrated more than a threefold increase in peak Na^+ current density from P0 to P8 while the Na^+ current density in the knockout mice remained constant over this period.

In neonatal rat spinal motoneurons, it is unclear which Na^+ channel isoform generates the I_{NaP} and whether the Na^+ channel subtype that generates the I_{NaP} and/or the I_{NaT} are the same or different. However, when one considers the electrophysiological data, it is possible that the channels generating I_{NaP} and I_{NaT} may be of the same subtype and that the I_{NaP} may be generated by a subpopulation of Na^+ channels that fail to inactivate or that have a more hyperpolarized membrane potential for activation than the Na^+ channels generating the I_{NaT} .

Some insight into the role different Na^+ channel isoforms may contribute to the I_{NaP}, I_{NaT}, or both comes from Purkinje cells. Three different Na^+ channel isoforms have

been identified in Purkinje cells, with each one contributing to the I_{NaP} to varying degrees. Vega-Saenz de Miera et al. (1997) suggest that $Na_v1.1$ generates the I_{NaT} and that $Na_v1.6$ is responsible for the I_{NaP}. Raman et al. (1997) using Purkinje cells isolated from mice lacking the $Na_v1.6$ isoform showed a 40% decrease in I_{NaT} and a 70% decrease in I_{NaP} compared to wild-type mice, indicating that while $Na_v1.6$ contributes to both currents, it is likely that other Na^+ channel isoforms also contribute to both the I_{NaT} and I_{NaP}. $Na_v1.1$, $Na_v1.2$, and $Na_v1.6$ express different levels of I_{NaP}. $Na_v1.2$ exhibits the lowest percentage at all levels of membrane depolarization, $Na_v1.1$ exhibits a large I_{NaP} that decreases with membrane potential depolarization, and $Na_v1.6$ exhibits a I_{NaP} that increases linearly with membrane potential depolarization (Goldin, 1999). It is also appears that the $Na_v1.6$ has a more depolarized membrane potential for activation compared to $Na_v1.1$ or $Na_v1.2$ (Catterall *et al.,* 2005).

In Purkinje cells it appears that all three of the aforementioned channels could contribute to the I_{NaP} with the majority of that current arising from $Na_v1.6$. Whether or not this applies to spinal motoneurons is unknown. It is also interesting to note that when $Na_v1.6$ channels are lacking ß subunit expression, the voltage dependence for their activation is depolarized compared to that of the $Na_v1.1$ or $Na_v1.2$. When the ß subunits are expressed all 3 isoforms have the same activation voltage (Smith *et al.,* 1998). These data may provide an explanation for the generation of both I_{NaT} and I_{NaP} in spinal motoneurons. In spinal motoneurons it is generally thought that the Na^+ channels generating the I_{NaP} are activated at a membrane potential ~10 mV more negative than those that generate the I_{NaT} responsible for the action potential (Crill, 1996). Perhaps the I_{NaT} is generated to a large extent by $Na_v1.6$ channels lacking the expression of their ß

subunits and that a subpopulation of $Na_v1.6$ channels expressing ß subunits provides the majority of current for the I_{NaP} with a correspondingly more hyperpolarized membrane potential.

Role of the Persistent Na^+ Current in Motoneuron Firing

Amplifying Synaptic Input

Based on data from different cell types, it is estimated that the I_{NaP} is activated about 10 mV negative to the I_{NaT} and makes up approximately 1% of the total Na^+ conductance (Crill, 1996). Although I_{NaP} is only a small fraction of the peak conductance, it is active over a wider voltage range than the I_{CaP} and may thus serve to amplify synaptic input (Schwindt & Crill, 1995; Stuart & Sakmann, 1995). Stuart and Sakmann (1995) used dendritic current injection in pyramidal neurons to demonstrate a non-linear increase in EPSP amplitude within the soma of the same neuron while Alzheimer et al. (1993) calculated that in a slice preparation the I_{NaP} could depolarize a hippocampal pyramidal cell at -50 mV membrane potential by 26 mV.

Repetitive Firing

The importance of I_{NaP} has also been implicated in repetitive firing (Lee and Heckman, 2001; Harvey et al., 2006). Lee and Heckman (2001) showed that deterioration of repetitive firing was associated with a decrease in a fast PIC. They suggested that due to the fast kinetics of the PIC, it was most likely generated predominantly by I_{NaP}, although the contribution of I_{CaP} was not eliminated. Correspondingly, Harvey et al. (2006) showed that motoneurons lacking the I_{NaP} were unable to fire rhythmically although transient firing could still be evoked.

Spike Initiation and Voltage Threshold

Based on the ability of I_{NaP} to amplify synaptic input and provide subthreshold depolarization (Schwindt & Crill, 1995; Stuart & Sakmann, 1995), I_{NaP} has been suggested to be a key element in depolarizing a cell to its threshold for spike initiation (Kuo *et al.*, 2006; Lee & Heckman, 1999). This is an important consideration for this thesis because PIC induction is a key feature of motoneuron physiology during fictive locomotion (McCrea and Rybak, 2007; Tazerart *et al.,* 2007; Tazerart *et al.,* 2008; Zhong *et al.,* 2007). It could be hypothesized that PIC induction leads directly to a hyperpolarization of V_{th} via effects on Na^+ channels. However recent findings indicate that PICs may not be key regulators of V_{th}. A dissociation between an enhancement of I_{NaP} and V_{th} hyperpolarization was evident in transgenic mice that develop amyotropic lateral sclerosis-like symptoms (Kuo et al, 2004, 2006). Kuo et al. (2004) investigated the electrophysiological properties of cultured spinal motoneurons from these presymptomatic mice and demonstrated hyperexcitability as indicated by an enhanced slope of the frequency/current (f/I) relation and increased maximal firing rate. However V_{th} was not affected. A subsequent study by Kuo et al. (2006) demonstrated an enhanced I_{NaP} in high input conductance cells. They used riluzole to block I_{NaP} and were unable to induce repetitive firing in cultured cells (presumably motoneurons) in response to slowly rising inputs. The ability of transient input to generate spikes was unaffected. Given that the experiments by Kuo and colleagues were performed on the same transgenic mice, it appears that increased motoneuron excitability resulting from an enhanced I_{NaP} does not necessarily lead to a hyperpolarization of V_{th}.

The importance of I_{NaP} in amplifying synaptic input, generating repetitive firing,

and depolarizing a cell to threshold during slowly rising inputs seems clear. Despite the apparent importance of PICs to motoneuron excitability, it remains unclear whether they play a significant role in V_{th} hyperpolarization or whether the effects of PICs would be limited to repetitive firing (Lee and Heckman, 2001; Harvey et al., 2006).

Afterhyperpolarization Modulation

Action potentials in motoneurons are followed by a hyperpolarization. This prolonged hyperpolarization is referred to as the AHP and is generated by a Ca^{2+} activated K^+ conductance (Sah, 1996). As the membrane potential depolarizes further from the resting potential, the AHP increases in amplitude because it moves further from its' own reversal potential (Coombs *et al.*, 1955). AHP amplitude can be used as a gauge of motoneuron excitability by referring to the f/I slope. During repetitive firing where several action potentials are generated in rapid succession, the AHP following the second spike is normally larger than that of the first spike. This is due to a 'summation' effect resulting from an incomplete activation of the K^+ conductance following the first spike; for the second spike, the K^+ conductance underlying the AHP would start at a higher level and reach a larger total conductance (Kernell & Sjöholm, 1973). This would lead to a decrease in motoneuron excitability evident as decrease in the f/I slope. Therefore, the AHP is important for determining the interspike interval during repetitive firing. This is true for motoneurons in a quiescent state, but what happens to AHP amplitude and the resulting f/I slope during a motor output?

Brownstone et al. (1992) investigated repetitive firing produced by intracellular current injection (control) and compared it with repetitive firing produced during fictive locomotion in the decerebrate cat. They demonstrated that the AHPs evoked at rest were

significantly larger than those produced at similar membrane potentials during fictive locomotion. Similar results were demonstrated during pharmacologically evoked locomotor-like activity using the *in vitro* neonatal rat spinal cord, indicating that AHP amplitude reduction can be mediated via an intraspinal mechanism (Schmidt, 1994). The next logical question is "what neuromodulatory system could be mediating this effect?" Recently Miles et al. (2007) using spinal cord slices from neonatal mice demonstrated that a decrease in AHP amplitude can be mediated via the cholinergic system, specifically via activation of the M_2-type muscarinic receptors. Even more interesting is that the origin of this cholinergic input is located in the spinal cord lateral to the central canal. Thus AHP amplitude can be altered during motor output in the absence of supraspinal input. The possibility that decreased AHP amplitude during locomotion results from a simple increase in motoneuron conductance during motor output or an active process decreasing the AHP conductance was addressed by Schmidt (1994). Schmidt (1994) concluded that an increase in conductance could not explain the decreased AHP amplitude because AHP reduction was also evident in instances when motoneuron conductance had actually decreased. Whether modulation of AHP amplitude during fictive motor outputs in the adult decerebrate cat is due to an increased conductance in spinal motoneurons has not been determined. It does appear, however, that the spinal cord has the circuitry necessary to modulate AHP amplitude during motor output.

Preparations to study V_{th} hyperpolarization

This thesis will further investigate V_{th} hyperpolarization using two main preparations, each with its own strengths. The first is an *in vitro* neonatal rat spinal cord preparation. In this preparation we will identify potential ionic mechanisms contributing

to V_{th} hyperpolarization. The second is a decerebrate cat preparation which will be used to determine whether V_{th} hyperpolarization occurs during fictive scratch. Fictive scratch is a rhythmic motor behaviour that can be evoked without electrical brainstem stimulation and in acute spinal animals.

The following sections provide a brief overview of the aforementioned preparations being utilized.

In vitro neonatal rat spinal cord

The motor system of the neonatal rat is immature, with rapid developmental changes occurring within the first 2 postnatal weeks. Although descending inputs to the lumbar spinal cord are present (Lakke, 1997), neonatal rats are unable to locomote effectively and explore their environments until the third postnatal week. In addition, the spinal motoneurons present are not fully developed morphologically or electrophysiologically (for review see Clarac *et al.*, 2004 or Vinay *et al.*, 2000). The following sections are summaries of information provided in reviews by Clarac et al., 2004 and Vinay et al., 2000, to address the utility of the *in vitro* preparation in motoneuron research.

During the postnatal period motoneuron R_{in} decreases with age, consistent with increased motoneuron size, although the contribution of changes in other membrane properties cannot be ruled out, such as a decrease in membrane resistivity. Corresponding with this data, motoneuron rheobase values increase more than fivefold. These changes in motoneuron properties all point to developmental decreases in motoneuron excitability, decreasing their responsiveness to synaptic inputs.

Additional changes in motoneuron properties include the continuing development of multiple ionic currents. The development of K^+ currents act to: reduce action potential duration by enhancing the slope of repolarizing phase, enhance the AHP, and enhance cell repolarization, thus facilitating repetitive firing (Vinay *et al.*, 2000). Ca^{2+} currents also increase during the perinatal time period, with the L-type Ca^{2+} current providing approximately 10% of the total Ca^{2+} current. This current has been suggested to increase following P7 in mice (Jiang *et al.*, 1999) and would thus have a large role in the development of PICs and repetitive firing. There are also changes in Na^+ currents during development. The lumbar motoneurons in the rat become excitable at embryonic day 15 and demonstrate a V_{th} more depolarized than at birth (-31 mV and -47 mV, respectively) without changes in resting membrane potential. Postnatally, there is also an increased slope of the rising phase for the action potential reducing the duration and enhancing motoneuron firing. These changes in action potential properties are likely due to a hyperpolarizing shift in the threshold for NaT channel activation and increased numbers of existing Na^+ channels and not the expression of new Na^+ channel subtypes (Gao & Ziskind-Conhaim, 1998).

Although the motor system of the neonatal rat is not fully developed, this preparation does offer several advantages for studying motoneurons in the lumbar spinal cord: (1) the surgery can be done quickly taking approximately 30 minutes in total. This includes anaesthesia, decerebration, evisceration, ventral laminectomy and removal of the spinal cord from the vertebral canal. The cord is then pinned to a Sylgard-plated petri dish, the dura removed and an incision made in the ventral cord to allow microelectrode penetration; (2) the central nervous systems neurons are unmyelinated and the spinal cord

is small in diameter, allowing a viable preparation to last hours provided the artifical cerebrospinal fluid (aCSF) is sufficiently bubbled with 95% O_2/5% CO_2 and the pH is maintained around 7.4; (3) manipulation of motoneuron excitability is done relatively easily by the addition of various pharmacological agents to the aCSF or by modulation of various ion concentrations in the aCSF such as K^+ (Clarac *et al.*, 2004).

Thus, while the *in vitro* neonatal rat preparation has its drawbacks, the ability to record from spinal motoneurons in a controlled environment and to be able to modulate their excitability via pharmacological or other means is a valuable tool in studying the excitability of spinal motoneurons.

Veratridine

The following section discusses the neurotoxin veratridine, which is used in combination with an *in vitro* neonatal rat spinal cord preparation to study the effects of Na^+ channel modulation on V_{th} in Paper 1 of this thesis.

Neurotoxins are commonly used to investigate ion channel function. They can be used to determine their physiological effects on cells such as motoneurons and to provide insights into the mechanisms through which various neuromodulators may affect excitability. Veratridine is an alkaloid found in the rhizomes of *Veratrum album* (Wang & Wang, 2003). Veratridine belongs to a group of lipid-soluble neurotoxins including barachotoxin, aconitine, and grayanotoxin, also used to study voltage-gated sodium channels. Veratridine is referred to as a partial Na^+ channel agonist and can cause hyperexcitability, convulsions, paralysis, and death (Wang & Wang, 2003). Generally, veratridine increases excitability by causing Na^+ channels to open more easily and prolonging their open time (Hille, 2001). The exact binding site for veratridine within the

Na^+ channel α-subunit is unclear. Wang and Wang (1998) have provided evidence for a receptor within the S6 segments of the Na^+ channel α-subunit using point mutations.

Effects on Excitable Membranes

Veratridine has multiple effects on the voltage-gated Na^+ channels including: (1) shifting in the midpoint of the activation curve to more hyperpolarized membrane potentials by 20 to 90 mV (Leibowitz *et al.*, 1986); (2) slowing channel inactivation which leads to a failure of Na+ channel inactivation producing what is known as a persistent "tail" current (Sutro, 1986); (3) reduced single channel conductance to about 25% of that of normal Na^+ channels (Barnes & Hille, 1988); and (4) reducing the normal strong selectivity for Na^+ ions. In addition to veratridine's effects on Na^+ channels, it also blocks voltage-gated K^+ channels (Verheugen *et al.*, 1994) and Ca^{2+} channels in neuroblastoma cells (Romey & Lazdunski, 1982).

The Decerebrate Cat

The Central Pattern Generator

In the early 1900's, the first evidence appeared that rhythmic movements could be elicited in animals void of input from the cortex or brainstem. Sherrington observed 'reflexive stepping' in a chronic spinal cat, suggesting that the circuits necessary for locomotion were present in the spinal cord. He postulated that the locomotor activity he observed was due to the activation of a chain of reflexes initiated by afferent input from the periphery. However Brown (1911) demonstrated that a locomotor rhythm, consisting of rhythmic, alternating contractions of the hindlimb muscles was possible in deafferented, spinal transected cats. Brown thus concluded that the locomotor rhythm did

not result from a chain of reflexes triggered by afferent input, but rather that the circuits necessary for locomotion were located within the spinal cord. Brown proposed a general model termed the "half centre" for the control of locomotion (Brown, 1914). This central programming of circuits within the spinal cord is now commonly referred to as the central pattern generator or "CPG" for locomotion (Stuart & Hultborn, 2008).

As was previously discussed, V_{th} hyperpolarization was first demonstrated during fictive locomotion, with subsequent work using an *in vitro* model of locomotor-like activity. A question that arises however is whether V_{th} hyperpolarization occurs during other motor behaviours. In Paper 2 of this thesis, fictive scratch is used as another motor output in which to investigate V_{th} modulation. This warrants a brief discussion regarding some commonalities and differences between locomotion and scratch in the adult cat. First however, a general description of each form of motor output is provided.

Cat Locomotion

As described by Rossignol (1996), locomotion in the cat involves two general phases of activity, swing and stance. During swing, the limb is lifted from the ground and propelled from a posterior to an anterior position. This occurs via flexion at the ankle, knee, and hip. Extension begins at the ankle and knee while the hip is still flexed before the paw contacts the ground. As the paw touches the ground, the knee and ankle are passively flexed as they begin weight support. Extension continues during stance as all joints are extended to propel the body forward. Swing and stance are further characterized by a strictly alternating pattern of muscle activation with flexors active during swing and extensors during stance. The more complex activation patterns of bifunctional muscles will not be discussed here (see Rossignol, 1996).

During real locomotion, the cycle duration can vary considerably, depending on locomotor speed. Changes in speed however are mainly determined by changes in the duration of the extensor phase, while the duration of flexor activity during the swing phase remains somewhat constant (Rossignol, 1996).

Cat Scratch

Early investigations by Sherrington (1903, 1906, 1910, and 1917) characterized scratching movements of decapitated and decerebrate cats and spinal dogs. Scratching is an example of a cyclical hindlimb motor behaviour different from locomotion. In the cat, it is an automatic response to cutaneous stimuli around the ear, head, or neck consisting of rapid rhythmic movements of the ipsilateral hindlimb in order to remove the stimuli. According to Kuhta and Smith (1990), the scratch reflex in intact adult cats consists of three phases, approach, cyclic, and return. The approach phase, wherein the ipsilateral hindlimb is positioned for scratching is initiated by the cat adopting one of three postural positions, lying, sitting, or standing. During this phase, there is an initial period of tonic flexion in the ipsilateral hindlimb as it is positioned toward the head combined with simultaneous extension of the contralateral hindlimb. This is followed by the cyclic or rhythmic phase characterized by strict and rapid alternation of flexors and extensors of the ankle, knee, and hip joints resulting in repetitive scratching of the irritated area of the skin. During the return phase the hindlimb is returned to the pre-response posture.

The efferent activity during the fictive scratch reflex (fictive due to neuromuscular block) is similar to that of real scratch with a cycle duration of ~250 ms, flexor phase ~200 ms, extensor phase ~50 ms (Berkinblit *et al.*, 1978). Scratch can also be elicited in deafferented preparations (Sherrington, 1910) although the hindlimb

movements are not as well coordinated and in some cases the paw cycles in the air without actually touching the ear. It can also be evoked following spinal transection rostral to C1 (Sherrington, 1903; Deliagina *et al.*, 1981). The activity of the muscles during fictive scratch remains very similar to that during normal movement (Deliagina *et al.*, 1975). It is thus evident that the mechanism producing scratching movements is located within the spinal cord.

Summary of Locomotion and Scratch in the Cat

While locomotion and scratch are different movements, they share common features including: (1) alternation of flexors and extensors, and (2) longer flexor than extensor phase of activity. Differences include: (1) scratching is a faster movement, thus having shorter cycle durations, and (2) locomotion is characterized by the movement of all four limbs, whereas scratch involves only one hindlimb.

The motoneuron activity pattern also differs between fictive locomotion and fictive scratch. For example, during fictive locomotion peroneous longus is active in phase with the flexors and active in phase with the extensors during fictive scratch. During fictive scratch, the activity pattern of posterior bicep and semintendinosous (PBSt) is similar to that of peroneous longus, with both largely active in phase with extensors. PBSt can display a variety of complex patterns of activity during fictive locomotion (Quevedo *et al.,* 2000), but will not be discussed here.

As was previously discussed, V_{th} hyperpolarization, AHP amplitude reduction, and the emergence of voltage dependent conductances all occur during fictive locomotion. Whether motoneurons are modulated in a similar fashion during fictive scratch is not known, although some evidence for the emergence of voltage-dependent

depolarizations during fictive scratch has been reported (see Figure 6B and C in Brownstone *et al.*, 1994). If in fact the modulation of these motoneuron properties is evident during fictive scratch, the next question is whether the changes are of a similar magnitude to that of fictive locomotion. This of course leads to the much bigger question of whether motoneuron excitability, in particular V_{th} hyperpolarization is a more general mechanism used by the motor system to enhance motoneuron firing during other motor outputs.

Evoking Fictive Scratch in the Decerebrate Cat

Fictive scratching in decerebrate cats can be elicited by mechanical stimuli applied to the skin around the ear, head, or neck or by electrical stimulation of the spinal cord at the C1-C2 segments both of which were used by Sherrington (1910). Deliagina (1975) showed that when electrical stimulation does not succeed, fictive scratch can be elicited by topical application of curare to the C1-C2 spinal segments followed by electrical stimulation. It is also possible to elicit fictive scratch in decerebrate cats by topical application of drugs such as curare (Deliagina *et al.*, 1975) or strychnine (Deliagina *et al.*, 1981) to the exposed dorsal roots of the C1-C2 spinal segments (Deliagina *et al.*, 1975; Feldberg & Fleischauer, 1960; Domer & Feldberg, 1960). Application of curare to the cervical cord of decerebrate, paralysed cats facilitates the scratch reflex by increasing sensitization to the mechanical stimulation (Domer & Feldberg, 1960).

The decerebrate cat preparation offers several advantages for studying spinal motoneuron excitability including: (1) a mature nervous system; (2) the ability to generate motor outputs in the absence of sensory or cortical input; and (3) the ability to

generate motor output following isolation of the spinal cord from descending input, including the brainstem after spinal transection. This preparation thus provides a means to study mature spinal motoneurons during a motor output in which they are isolated from sensory and supraspinal input, being driven only by the spinal CPG.

Research Goals

The overall goal of this thesis is to further characterize V_{th} hyperpolarization from a mechanistic and functional point of view. **More specifically, the goals of this thesis are to: (1)** understand the ionic mechanisms by which V_{th} hyperpolarization occurs through pharmacological modulation of voltage-gated Na^+ channels; and **(2)** determine whether V_{th} hyperpolarization occurs during motor behaviours other than locomotion, is dependent upon brainstem stimulation, or requires intact brainstem and spinal cord connections.

Paper 1

To address our first goal, we made intracellular recordings from spinal motoneurons using the *in vitro* neonatal rat spinal cord preparation to determine whether modulation of Na^+ channels could induce V_{th} hyperpolarization. We compared the V_{th} of motoneurons in the absence (control) and presence of veratridine, a potent Na^+ channel agonist. Because of the space clamp issues associated with intracellular recordings in this preparation, we used human embryonic kidney (HEK) cells expressing the $Na_v1.2$ channel subtype to confirm that veratridine was in fact modifying Na^+ channels in a manner consistent with previous findings in other cell types (see previous section for details). The $Na_v1.2$ channel subtype was used because it is one of the main Na^+ channels expressed in the developing neonatal rat spinal cord (Goldin, 2001) used in the whole cord preparation.

Specific hypotheses:

1) **A hyperpolarizing shift in voltage-gated sodium channel activation would cause spinal motoneuron V_{th} hyperpolarization**
2) **Spinal motoneuron V_{th} hyperpolarization does not require the enhancement of a persistent inward current**

The results show that modulation of Na^+ channels does induce V_{th} hyperpolarization through a negative shift in the voltage dependence of activation of voltage-gated Na^+ channels and does not require the enhancement of a persistent inward current.

Paper 2

To address our second goal, whether motoneuron excitability is enhanced during fictive scratch by a hyperpolarization of V_{th} or a reduced AHP amplitude, we made intracellular recordings from the spinal motoneurons of the decerebrate cat. This preparation provides a means to study spinal motoneurons during a motor output in which they are isolated from sensory and supraspinal input, being driven only by the central pattern generator. An additional advantage of using this preparation is the ability to generate fictive scratch following isolation of the spinal cord from descending input, including the brainstem via spinal transection. Thus, in two cats we performed an acute spinal transection at the C1 segment to isolate the spinal cord from descending input.

Specific hypotheses:

1) **V_{th} hyperpolarization is evident during fictive scratch**
2) **V_{th} hyperpolarization does not require electrical brainstem stimulation**
3) **V_{th} hyperpolarization does not require an intact spinal cord**
4) **AHP amplitude is reduced during fictive scratch**

The results show that V_{th} hyperpolarization and AHP amplitude reduction occur in the absence of electrical brainstem stimulation during fictive scratch, do not require motoneuron firing, and occur following spinal transection. The significance of these observations include: (1) the first demonstration of an intraspinal mechanism for regulating threshold properties in spinal motoneurons and (2) further evidence for intraspinal mechanisms capable of reducing AHP amplitude.

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II. Paper 1: Modulation of voltage-gated sodium channels induces voltage threshold hyperpolarization in spinal motoneurons

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Running Head: Sodium channel-mediated voltage threshold hyperpolarization

Keywords: voltage threshold, motoneuron, sodium currents

Abstract

Previous work has shown that motoneuron excitability is enhanced by a hyperpolarization of the membrane potential at which an action potential is initiated (V_{th}) at the onset and throughout brainstem evoked fictive locomotion in the adult decerebrate cat and neonatal rat. Because the conductances mediating V_{th} hyperpolarization are unknown, this study sought to determine if the modulation of voltage-gated sodium channels could induce V_{th} hyperpolarization. Blind patch whole-cell recordings were made from antidromically identified lumbar spinal motoneurons in an isolated neonatal rat spinal cord preparation. Recordings were made with and without the bath application of veratridine, a sodium channel agonist. Veratridine induced a significant hyperpolarization of V_{th} in all 29 motoneurons examined (mean hyperpolarization -6.6 ± 4.3 mV). Veratridine also significantly increased the amplitude of persistent inward currents (PICs) (mean increase 72.5 ± 98.5 pA) evoked in response to depolarizing current ramps. The effects of veratridine on V_{th} and PIC amplitude had different time courses such that V_{th} hyperpolarization occurred within the first minute of veratridine application while PIC amplitude facilitation required between 5 and 10 minutes. Veratridine also produced a negative shift in the activation voltage of $Na_v1.2$, β1 subunit channels expressed in HEK-293 cells. We suggest that central neuronal circuitry in mammals could affect V_{th} in manner similar to that of veratridine by inducing a negative shift in the activation voltage in sodium channels. Furthermore, this shift would not be dependent upon an enhancement of PICs.

Introduction

The electrical properties of spinal motoneurons are reconfigured to facilitate firing during motor output. For example, during fictive locomotion in the decerebrate cat, motoneurons exhibit a reduction of rheobase current (Krawitz *et al.*, 2001), a decreased afterhyperpolarization amplitude (Brownstone *et al.*, 1992; Schmidt, 1994), and the emergence of persistent inward currents (PICs) (Brownstone *et al.*, 1994), all of which enhance motoneuron firing. At the onset of fictive locomotion evoked by brainstem stimulation in adult decerebrate cats the membrane potential at which an action potential is initiated, referred to as the voltage threshold (V_{th}), becomes hyperpolarized (Krawitz *et al.*, 2001). The effect on V_{th} was robust (mean change of -8.0 mV), occurred in every motoneuron examined, and recovered within seconds to minutes of locomotor cessation. Subsequent studies have demonstrated V_{th} hyperpolarization during brainstem evoked locomotor-like activity in neonatal rats (Gilmore & Fedirchuk, 2004) and during fictive scratch in the adult decerebrate cat (see Paper II of this thesis). These results indicate that V_{th} hyperpolarization may be a mechanism that is common across mammalian species and used by the motor system to increase motoneuron excitability during rhythmic motor behaviours. Mechanistically, the descending serotonergic pathways (Gilmore & Fedirchuk, 2004) and exogenous application of the monoamines serotonin (5-HT) or noradrenaline (NA) (Fedirchuk & Dai, 2004) are capable of inducing V_{th} hyperpolarization. Additional data from our lab have demonstrated that the V_{th} hyperpolarization during fictive scratch in cat persists following spinal transection at the first cervical region and therefore does not require intact descending projections, implicating an intraspinal mechanism capable of inducing motoneuron V_{th}

hyperpolarization in addition to the descending monoaminergic pathways (see Paper II of this thesis). The ionic mechanism(s) through which neuromodulators may act to account for V_{th} hyperpolarization have yet to be determined.

Given the numerous channels and conductances present in spinal motoneurons (for review see Rekling *et al.*, 2000), an obvious potential mechanism for V_{th} hyperpolarization is a modulation of the properties of voltage-gated Na^+ channels (VGSCs). Because the fast-inactivating, transient Na^+ current (I_{NaT}) plays a central role in the initiation and propagation of action potentials in neurons, VGSCs are ideally situated as targets for the neuromodulation of neuronal excitability. Such actions have been demonstrated in isolated hippocampal neurons during application of dopamine and acetylcholine (Cantrell *et al.*, 1997; Cantrell *et al.*, 1996). In addition to I_{NaT}, spinal motoneurons can display PICs generated by non-inactivating or slowing inactivating VGSCs and voltage-gated Ca^{2+} channels activated at membrane potentials more depolarized than at rest (Lee & Heckman, 1998). Of these currents, the slowly inactivating or "persistent" Na^+ current (I_{NaP}) plays an important role in the gain of the neuronal input-output relationship, contributing approximately 40% of the total PIC in rat sacral motoneurons (Harvey *et al.*, 2006b). I_{NaP} has been shown to be important for amplifying synaptic input in the neocortex (Schwindt & Crill, 1995; Stuart & Sakmann, 1995), inducing repetitive firing in cat lumbar motoneurons (Lee & Heckman, 2001), and implicated in spike initiation during slowly rising inputs (Kuo *et al.*, 2006; Lee & Heckman, 1999). Whether I_{NaP} contributes to V_{th} modulation during transient synaptic events has not been examined.

To identify putative mechanisms of V_{th} hyperpolarization, computer modeling

demonstrated that the most effective means to induce V_{th} hyperpolarization was through modulation of the I_{NaT} underlying action potentials, specifically by increasing the Na^+ channel conductance by 50% or by hyperpolarizing its voltage dependency for activation by 3 mV (Dai *et al.*, 2002). Modelling however suggested that enhancement of I_{NaP} was an unlikely candidate for V_{th} hyperpolarization since large changes in I_{NaP} had large effects on action potential shape with only modest hyperpolarization of the V_{th} (Dai *et al.*, 2002).

To investigate the possible role of Na^+ channel modulation as a mechanism of V_{th} hyperpolarization, the present study employed veratridine. Veratridine is a Na^+ channel agonist, which has multiple effects on the VGSCs of cells including: (1) a shift in the midpoint of the activation curve by 20 to 90 mV to more hyperpolarized membrane potentials (Leibowitz *et al.*, 1986); (2) a slowing of inactivation leading to a persistent tail current (Sutro, 1986); (3) a reduction of single channel conductance to about 25% of that of normal Na^+ channels (Barnes & Hille, 1988); and (4) an increased I_{NaP} amplitude (Tazerart *et al.*, 2008). Based on the results of a previous computer modeling study (Dai *et al.*, 2002) we hypothesize that: (1) a hyperpolarizing shift in VGSCs activation will cause spinal motoneuron V_{th} hyperpolarization, and (2) V_{th} hyperpolarization can occur independently of an enhancement of PICs in spinal motoneurons.

To address these hypotheses we utilized two preparations. The first was the *in vitro* neonatal rat, spinal cord, preparation in which veratridine was added to the bathing solution to modulate VGSCs. The second preparation used human embryonic kidney (HEK) cells expressing the $Na_v1.2$ channel to confirm the functional effects of veratridine on this isolated Na^+ current. The $Na_v1.2$ channel was chosen because it is likely the main

isoform present in the rodent spinal cord during the first postnatal week (Beckh *et al.*, 1989; Felts *et al.*, 1997).

Methods

In vitro neonatal rat spinal cord

Surgical procedures

Experiments were conducted on whole spinal cords isolated from neonatal (postnatal day 1-5) Sprague-Dawley rats. The animals were anesthetized with halothane in a chamber, decapitated, eviscerated, and placed in cooled (4°C), modified artificial cerebrospinal solution (mACSF - described below) aerated with 95% O_2 - 5% CO_2 for dissection. Ventral spinal laminectomy was done with fine scissors and the spinal roots were cut near the dorsal root ganglia. The spinal cord was then removed and pinned to a dish with a Sylgard bottom. The dura was removed and the spinal cord transected at T9. The isolated thoracolumbar cord was then pinned ventral side up and pial incisions were made using an etched Tungsten electrode at the L2 and L5 levels to allow electrode impalement. The mACSF was then replaced by normal artificial cerebrospinal fluid (nACSF) containing synaptic blockers (described below) and allowed to slowly warm to room temperature (~22°C) prior to recordings. All procedures were conducted with the approval of the University of Manitoba animal care protocol review committee and in accordance with guidelines for the ethical treatment of animals issued by the Canadian Council on Animal Care.

Intracellular recording

Motoneurons of the lower lumbar segments (L2 and L5) were targeted for single-cell patch recordings using glass microelectrodes pulled with a Narishige PP-83 two-stage puller and filled with a solution containing 140 mM potassium gluconate, 0.2 mM EGTA, 10 mM Hepes, and KOH to bring the pH to 7.3. The filled microelectrodes had resistances ranging from 3.8 to 4.5 MΩ. 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 electrical stimulation of the ventral root (200-500 μA) corresponding to the spinal segment containing the neuron being recorded was done in an attempt to activate the neuron antidromically. Only data from antidromically activated neurons, which are considered motoneurons, is reported in the present study. An Axopatch 1D microelectrode amplifier controlled the pCLAMP 9.2 software (Axon Instruments) used for recording. Series resistance was monitored to ensure it did not change significantly during the recording period and was always ≤ 40 MΩ. Neither series resistance nor tip potentials were compensated.

Solutions and chemicals

Two types of ACSF were used in these experiments: mACSF was used during dissection to minimize potential excitotoxicity and normal (n) ACSF was used during recording. The mACSF contained (in mM): NaCl (25), sucrose (188), KCl (1.9), NaH_2PO_4 (1.2), $MgSO_4$ (10), $NaHCO_3$ (26), kynurenic acid (1.5), glucose (25), and $CaCl_2$ (1.0). The nACSF contained (in mM): NaCl (125), KCl (2.5), $NaHCO_3$ (26),

NaH_2PO_4 (1.25), D-Glucose (25), $MgCl_2$ (1), $CaCl_2$ (2.5). The pH of these solutions was adjusted to 7.3 with KOH. The following synaptic blockers were added in μM concentrations to the nACSF to reduce synaptic noise in the recordings: (20) AP5, (10) CNQX, (10) bicuculline, and (10) strychnine. In some experiments, 10 mM TEA and 5 mM 4-AP were also added to facilitate the expression of PICs by a blockade of K^+ channels. Veratridine (30 μM) was applied from a 10 mM stock solution to a small-volume (≤ 8 ml) bath aerated with 95% O_2 - 5% CO_2.

Measurement of V_{th}

Initial experiments compared V_{th} in current-clamp and voltage-clamp recordings from the same motoneurons. V_{th} was used to describe the generation of an action potential in current-clamp recordings, or the fast transient inward current in voltage-clamp. The fast inward current generated during voltage-clamp should be dominated by the same current underlying action potentials recorded in current-clamp. In current-clamp, V_{th} was defined as the membrane potential at which depolarization increased at ≥ 10 Vs^{-1}. At the 10 kHz sampling rate used, the V_{th} value was the membrane potential of the data point that was ≥ 1 mV more depolarized than the preceding point. In voltage-clamp, V_{th} was defined as the minimum depolarizing step which elicited a fast inward current. The effects of veratridine on V_{th} were consistent in direction between cells in which V_{th} was measured in both current-clamp and voltage-clamp, although the amount of change could be different. It was determined that V_{th} measurements made in voltage-clamp were more stable and faster to determine than those obtained in the current-clamp. Thus, voltage-clamp was used to determine all V_{th} values reported in this study.

V_{th} was determined using a voltage-clamp protocol as was done previously (Fedirchuk & Dai, 2004). An initial holding potential of -60 mV was used to approximate the resting membrane potential and 200 ms depolarizing pulses were successively delivered in +2 mV increments. Steps were delivered at a repetition rate of 2 Hz. The potential of the smallest depolarizing step able to elicit a fast transient inward current, which would have mediated an action potential is considered V_{th}. V_{th} values determined with this method were stable for prolonged periods. A hyperpolarization of the V_{th} was manifested as a smaller depolarizing voltage step (i.e. more negative membrane potential) being able to induce a fast transient inward current. During recording, the protocol for measuring V_{th} was repeated three to four times in control and every 30-60 seconds following veratridine administration for up to 20 minutes. The V_{th} data reported are averages of 2-3 trials in each condition (control and veratridine). Voltage-clamp recordings from motoneurons in our whole cord preparation are subject to the limitations of "space clamp" issues. That is, one cannot assume that the entire cell, including the soma and dendrites, is clamped at the same membrane potential.

Measurement of PIC amplitude and voltage dependency

PICs were recorded by a symmetrical slow voltage ramp of 10 s duration from a holding potential of -70 mV to a peak voltage of +10 mV (16 mV/s). Leak current was subtracted before calculation of PIC amplitude and $V_{1/2}$. Details of the calculations are illustrated in Figure 1. The PIC evoked on the ascending phase of the voltage ramp was used for calculating PIC parameters. To measure PIC amplitude a straight line was drawn along the horizontal current trace and an average of the points prior to where the straight

line is tangent to the current trace was defined as PIC baseline (Ibase). A 350-400 ms average of the lowest points (negative deflection in Fig. 1) on the current trace was defined as the maximal PIC amplitude (Imax). The amplitude of the PIC was calculated as the difference between Ibase and Imax (i.e. PIC amplitude = Imax-Ibase).

To measure the voltage dependency of PICs, initial analysis included the voltage of PIC onset and PIC $V_{1/2}$. PIC onset was defined as the last point where the horizontal line was tangent to the current trace, and the corresponding voltage on the command ramp was defined as the PIC onset voltage. A Boltzman charge-voltage equation (see equation 1 in legend of Fig. 1) was used to determine PIC $V_{1/2}$ as a measure of PIC voltage dependency. PIC $V_{1/2}$ represents the voltage at which half of the maximal current amplitude is attained. Comparison of multiple measures on the same data files indicated that PIC $V_{1/2}$ was a more reliable measure of PIC voltage dependency and was thus used instead of PIC onset voltage in the present study. As was the case for V_{th} measurements, 3-4 PIC recordings were made in control and every 30-60 seconds following veratridine administration for up to 20 minutes. PIC amplitude and $V_{1/2}$ values reported are an average of 2-3 trials in both conditions (control and veratridine).

Recombinant cells

In additional experiments, recordings were made from HEK-293 cells stably over-expressing the $rNa_v1.2$, $\beta1$ subunit combination or the $hCa_v1.2$, $\beta3$, $\alpha2\delta$ combination that were cultured using techniques previously described by Ilyin et al. (2005). For electrophysiology, the cells were plated on poly-D-lysine coated 35 mm Petri dishes and maintained in Dulbecco's Modified Eagles Media with 2 mM L-glutamine, and 10% fetal

bovine serum in a 5% CO_2 environment at 37° C for 24-48 hours before recordings were made.

Electrophysiology

On the day of the experiment the culture medium was replaced with external recording solution which was continually perfused through the Petri dish via a gravity-driven perfusion system. The various concentrations of veratridine were applied to the cell of interest through individual barrels of a fast superfusion system located <100 μm from the cell. Cells were visualized with a Nikon TE2000-U inverted microscope with whole-cell patch clamp recordings made with an Axopatch 200B amplifier, 1322A A/D converter and pClamp software (v. 8; Molecular Devices, Union City, CA) and data stored on a personal computer. Sodium currents were low-pass-filtered at 5 kHz and digitized at 10 kHz while Ca^{2+} currents were sampled at 5 kHz. Borosilicate patch pipettes had resistance values between 1.4 – 1.8 MΩ when filled with intracellular recording solution. Series resistance was monitored throughout the experiment and compensated between 60-80% to ensure a voltage error of less than 5 mV. No adjustments were made for the liquid junction potentials.

Solutions

To record Na^+ currents the intracellular solution contained (in mM): CsF (140), NaCl (10), $CaCl_2$ (1), $MgCl_2$ (5), EGTA (11), glucose (10), HEPES (10), ATP (4), GTP (0.5), with pH adjusted to 7.2 with CsOH. The external recording solution contained (in mM): NaCl (150), KCl (5.4), $CaCl_2$ (1.8), $MgCl_2$ (1), HEPES (5), pH = 7.4 with NaOH. Calcium currents were recorded with an internal solution containing (in mM): CsCl

(134), $MgCl_2$ (3), EGTA (10). HEPES (40), ATP (4), GTP (0.5), pH = 7.2 with CsOH. For calcium current recording the extracellular recording solution contained (in mM): NaCl (137), KCl (2.7), $MgCl_2$ (1), NaH_2PO_4 (0.4), glucose (5.5), $NaHCO_3$ (12), $CaCl_2$ (12), HEPES (10), pH = 7.4 with NaOH.

Data analysis

Data were analyzed in Clampfit 9.2 (Axon Instruments, Foster City, CA). Only one cell per rat was utilized as the complete washout of veratridine was unattainable in the whole cord preparation. Unless otherwise specified, a paired Student's t-test was used to test for statistical differences before and after veratridine administration, with a significance level of $P < 0.05$. Data sets that did not pass a normality test underwent the nonparametric Wilcoxon Signed Rank Test, with a significance level of $P < 0.05$.

Results

***In vitro* neonatal rat spinal cord**

Veratridine induces hyperpolarization of motoneuron V_{th}

Veratridine elicited a significant (paired t-test; $P \leq 0.001$) hyperpolarization of the V_{th} in all 29 motoneurons (range: -2 to -16 mV; mean: -6.6 mV). Figure 2 illustrates a representative example of the effect of veratridine on the V_{th} of an L5 motoneuron in a P5 neonatal rat. As shown in Fig. 2A, depolarizing steps increasing in +2 mV increments were delivered from a holding potential of -60 mV. Three depolarizing steps are shown in the bottom panel with the current responses in the top panel. The first fast inward current was elicited at a step to -32 mV (indicated by arrow). Depolarizing voltage steps to -34 mV and -30 mV were subthreshold and suprathreshold, respectively. Further step

depolarizations resulted in additional fast inward currents (data not shown). Within 1 minute of the addition of 30 μM veratridine to the bath, the first fast inward current was elicited at -36 mV (Figure 2B). Cell current responses to subthreshold (-38 mV) and suprathreshold (-34 mV) depolarizing steps are shown as in 2A. This difference represents a -4 mV hyperpolarization of the V_{th}.

The minimum concentration of veratridine able to reproducibly hyperpolarize V_{th} was 30 μM. Titration of the veratridine concentration showed that concentrations of 10 μM produced V_{th} depolarization (5/5) and a concentration of 20 μM could produce V_{th} hyperpolarization (2/3) or depolarization (1/3). The change in V_{th} for each motoneuron following bath application of 30 μM veratridine is illustrated in Figure 3. In 27/29 cells, the amplitude of the fast inward current evoked by the +2 mV depolarizing steps was decreased significantly ($P < 0.001$; mean: 0.641 ± 0.896 nA; range: 0.022 to 3.665 nA) following veratridine application to the bath. Following veratridine administration to the bath, the initial fast inward current elicited via step depolarizations was accompanied by an increased tail current. In the whole cord preparation the tail current was proportional to the leak current observed at the onset of subsequent step depolarizations.

The effect of bath applied veratridine on V_{th} was assessed for 29 antidromically identified spinal motoneurons recorded in the L2 and L5 segments. All motoneuron recordings (29/29) were made in the presence of synaptic blockers (AP5, CNQX, bicuculline, and strychnine) in 13 of these recordings K^+ channel blockers (TEA and 4AP) were also present (see Methods). There was no significant difference between the change in V_{th} recorded in solution containing synaptic only and synaptic + K^+ channel

blockers (P = 0.08). Therefore, all motoneurons were grouped together for further analysis, regardless of the blockers used. Each cell served as its own control.

The input resistance (R_{in}) of all cells (n = 29) was measured before and after veratridine administration. In 23/29 cells, the R_{in} was reduced significantly within the first minute of veratridine application to the bath (P = 0.019; mean: 149 ± 322.8 MΩ). There was no relation between the amount of V_{th} change and R_{in} change (Pearson product moment correlation; r = -0.04, P = 0.88). There was no relation between the magnitude of the veratridine effect on V_{th} and the age of the neonatal rat from which the motoneuron recording was made (Pearson product moment correlation; r = 0.25, P = 0.19; not illustrated).

Veratridine increases PIC amplitude

Figure 4 illustrates a representative example of the veratridine induced enhancement of the PIC amplitude. As previously mentioned (see Methods) a symmetrical voltage bi-ramp (bottom trace) was applied to assess PIC amplitude on the ascending portion of the ramp. In this example there was a facilitation of PIC amplitude in the presence of 30 μM veratridine (red trace: 180.6 pA) when compared to control (black trace: 40.7 pA). K^+ channel blockers (TEA and 4AP) had been added to the bath.

All data was recorded in the presence of synaptic blockers. In 10 cells, K^+ channel blockers were also administered to the bathing solution. There was no significant difference (P = 0.3) between the amplitude of the PIC in cells with or without K^+ channel blockers present. Therefore, the data was grouped together for further analysis. By 10 minutes after bath application, veratridine significantly (P = 0.01) increased the total PIC

amplitude (mean: 72.5 pA ± 98.5 pA). However when the time course of the PIC amplitude change was assessed shortly after veratridine administration, the effect on the PIC could be either an increase or decrease in amplitude. Figure 5 shows the percentage change in PIC amplitude versus the absolute change in V_{th} over a 15 minute period in one cell. In this example, PIC amplitude initially decreases following the addition of 30 μM veratridine to the bath. This is followed by an increase in PIC amplitude after ~ 9 minutes. Experiments in which an initial decrease in PIC amplitude was followed by an increase in PIC amplitude (n = 4) showed that the facilitation was evident by the 10 minute mark, so that determination of the direction of veratridine effect was made after that time for that particular cell. Measurements of PIC amplitude in the presence of veratridine were made after a minimum of 10 minutes exposure to ensure that PIC amplitude facilitation had occurred, but may not represent the maximal effect. The amplitude of the PIC facilitation could continue to increase, however there was no further enhancement of V_{th} hyperpolarization.

Veratridine has variable effects on PIC voltage dependency

Veratridine had variable effects on PIC voltage dependency, causing either a depolarization (n = 9/17) or hyperpolarization (n = 8/17) of PIC $V_{1/2}$. Figure 6A1 is an example showing the PIC amplitude (top traces) under control condition (black trace) and in the presence of veratridine (red trace). Figure 6A2 is an expanded version of the boxed area in 6A1. The veratridine current trace has been normalized to the control trace. Arrows indicate the membrane potential in the voltage command (bottom trace, Fig 6A1) at which half of the maximal current amplitude was obtained. As shown in Fig. 6A2, PIC

$V_{1/2}$ was measured as -39.8 mV during control (black trace). Approximately 10 minutes after veratridine administration, PIC $V_{1/2}$ depolarized by 12.5 mV to -27.3 mV (red trace). Figure 6B1 is an example from a different cell again demonstrating an enhanced PIC amplitude in the presence of veratridine. However as shown in Figure 6B2, the PIC $V_{1/2}$ was measured as -38.4 mV during control and -42.1 mV after approximately 10 minutes of veratridine administration. This represents a hyperpolarization of PIC $V_{1/2}$ by 3.7 mV. V_{th} hyperpolarization occurred in both cells (i.e. by 4 mV in Fig 6A and 5.3 mV, in Fig 6B). Hyperpolarization of motoneuron V_{th} in the presence of veratridine was not clearly related to a hyperpolarization of PIC $V_{1/2}$.

Veratridine effects on V_{th} and PIC amplitude have different time courses

Even in cells in which veratridine caused an enhancement of the PIC after 10 minutes, when assessed at earlier time points, veratridine could induce a decrease in PIC amplitude (n = 4). Throughout the time period when the effect on the PIC amplitude reversed, there was a consistent hyperpolarization of V_{th} that was evident within 1-2 minutes of veratridine administration. Figure 5 shows normalized PIC amplitude and V_{th} for one of these cells over a 15 minute duration. Veratridine initially decreased PIC amplitude for up to 6 minutes post administration, which changed into a large facilitation (8-15 minutes). V_{th} was hyperpolarized by -2 mV within 2 minutes of veratridine application and reached its maximum level of -4 mV at 6 minutes. Therefore, the veratridine effect on the PIC amplitude appears to be independent of the V_{th} hyperpolarization.

Effects of Ca^{2+} antagonists on PIC amplitude and V_{th}

Since the PIC reported above could be comprised of both Na^+ and Ca^{2+} currents, we sought to determine whether we could attribute any of the veratridine effects on the PIC to an effect on Na^+ channels. Therefore the Ca^{2+} channel blocker Cd^{2+} (500 μM) was administered to the bathing solution (n = 5). Figure 7A illustrates an example of PICs evoked by a voltage ramp during control (black trace), in the presence of Cd^{2+} (blue trace), and following veratridine administration (red trace). Figure 7B is an expanded view of the boxed region in 7A. Figure 7B shows that administration of Cd^{2+} (500 μM) to the bathing solution decreased PIC amplitude, in this example to 30% (16.4 pA) of control. Administration of veratridine (30 μM) to the bath following Cd^{2+} administration resulted in a partial recovery of PIC amplitude to 64% of control (15.3 pA) (Fig. 7B). The V_{th} in the same cell was -32 mV in control and did not change following Cd^{2+} administration. In the presence of Cd^{2+}, veratridine hyperpolarized V_{th} by 8 mV (V_{th} = -40 mV), compared to control and Cd^{2+} conditions (data not shown).

In the absence of veratridine, Cd^{2+} decreased PIC amplitude by 56% (mean: 16.4 ± 4.76 pA; range: 9.4 to 22.8 pA) without affecting V_{th} (data not shown). Veratridine administration in the presence of Cd^{2+} resulted in an enhancement of the PIC within 5-10 minutes. In 2 experiments, the Na^+ channel blocker TTX (2 μM) was applied following Cd^{2+} and blocked the remaining portion of the PIC. An additional 2 experiments were conducted with 0 Ca^{2+} in the recording aCSF (data not shown). In both experiments (n = 2), veratridine induced hyperpolarization of the V_{th} (mean: -7.5 mV). Thus, V_{th} hyperpolarization occurred in the presence of synaptic and/or K^+ channel blockers, following Ca^{2+} channel blockade (500 μM Cd^{2+}), and in the absence of Ca^{2+} in the ACSF.

Recombinant Cells

Data was subsequently recorded from recombinant cells to confirm that the effects of veratridine on motoneuron excitability seen in the *in vitro* neonatal whole cord preparation were in fact due to modulation of Na^+ channel conductances.

Veratridine reduces peak current through $Na_v1.2$ sodium channels, but enhances PIC

To confirm that the effects of veratridine were mediated through Na^+ channel modulation, we used HEK cells that expressed the $Na_v1.2$ channel isoform (Figure 8). As seen in Fig. 8*A*, the veratridine application resulted in a reversible inhibition of the peak Na^+ current across all voltages. By examining the raw current traces it can be seen that, in addition to the reduction in peak current, veratridine caused a reversible *increase* in the non-inactivating component of the current during a voltage step (Fig. 8*B*; n = 9). This change in the ability of the channels to inactivate resulted in the expression of large, slowly deactivating tail currents at the termination of the voltage steps (Fig. 8*B*). In response to slow ramp stimulations, veratridine significantly enhanced the inward current on both the depolarizing and repolarizing limbs of the ramp (n = 8). These veratridine-enhanced responses to slow inputs were concentration dependent and reversible (Fig. 8C; n = 3). Finally, veratridine modification resulted in a hyperpolarization of the voltage-dependence gating compared to un-modified channels (Fig. 8D; n = 4).

Veratridine effects on L-type calcium channels

In addition to veratridines effects on Na^+ channels, there is evidence from neuroblastoma cells that veratridine blocks voltage-gated K^+ (Verheugen *et al.*, 1994) and

Ca^{2+} channels (Romey & Lazdunski, 1982). According to our previous modeling study (Dai *et al.*, 2002), a block of K^+ channels may also contribute to V_{th} hyperpolarization. Given that rodent spinal motoneurons express somatically located L-type Ca^{2+} channels of the α1c subtype (Jiang *et al.*, 1999) during the first week of postnatal development and that these channels are known to mediate a PIC (Fox *et al.*, 1987), we sought to determine whether veratridine was modulating currents other than Na^+ that could affect the V_{th}, thus we administered veratridine to HEK cells expressing L-type Ca^{2+} channels. This resulted in a reduction in the peak current amplitude during step depolarizations (38 % +/- 12; mean +/- SD; n = 6) but unlike the effect on Na^+ channels, was not accompanied by a shift in the voltage-dependence of activation or a change in tail-current kinetics (n = 6; data not shown). Furthermore, in the presence of 30 μM veratridine there was a *decrease* in the ramp current amplitude (37 % +/- 9; mean +/- SD; n = 3; data not shown) that was partly reversible.

Discussion

The most important finding is this study is that modulation of VGSCs induces V_{th} hyperpolarization in spinal motoneurons. This effect is mediated through modulation of the I_{NaT} responsible for the upswing of the action potential initiation and not by a I_{NaP}. Three main findings regarding these conclusions include: (1) veratridine induced activation of VGSCs at more negative membrane potentials results in a V_{th} hyperpolarization; (2) veratridine enhances the amplitude of PIC produced in spinal motoneurons and cultured neurons expressing $Na_v1.2$ channels; (3) there is no relation between the veratridine induced hyperpolarization of V_{th} and the enhancement of the amplitude or $V_{1/2}$ of PICs. These findings are in general agreement with previous studies that have demonstrated veratridines' ability to induce hyperexcitability and depolarization of excitable membranes ascribed to alterations in Na^+ channel properties.

Modulation of VGSCs induces V_{th} hyperpolarization in spinal motoneurons

The present results demonstrate a Na^+ channel mediated V_{th} hyperpolarization in spinal motoneurons. Data from our HEK cells expressing $Na_v1.2$ channels indicate that the voltage dependence of activation of veratridine modified Na^+ channels is shifted in the hyperpolarized direction. Given that the ventral horn of rat spinal cords express the mRNA for the $Na_v1.2$ channel subtype it is possible that a shift in activation kinetics of this Na^+ channel subtype is mediating the V_{th} hyperpolarization demonstrated in spinal motoneurons using the *in vitro* preparation. Of course effects on other Na^+ channel subtypes cannot be excluded. A Na^+ channel mediated V_{th} hyperpolarization in spinal motoneurons is in agreement with results from our computer modeling study. Dai and

colleagues (2002) used models of motoneurons with properties resembling those of real motoneurons *in vivo*. Of the 10 active conductances in the model, it was concluded that the most likely mechanisms contributing to V_{th} hyperpolarization were through the modulation of the Na^+ and K^+ conductances underlying spiking. More specifically by: (1) increasing initial segment Na^+ conductance by 50%; (2) hyperpolarization of the initial segment voltage dependency for Na^+ conductance; (3) 70% reduction in initial segment conductance generated by a K^+ conductance (delayed rectifier); or (4) depolarization of the initial segment voltage dependency for the delayed rectifier K^+ conductance. However they further suggested that of these conductance changes, the most likely means of V_{th} hyperpolarization was by increasing initial segment Na^+ channel conductance by 50% or by a hyperpolarization of its voltage dependency. The latter hypothesis is in agreement with our current results. Although we cannot rule out additional contribution of K^+ and/or Ca^{2+} channels in mediating V_{th} hyperpolarization, the fact that V_{th} hyperpolarization occurred and was of similar magnitude in the presence of K^+ and Ca^{2+} channel blockers, indicates that the modulation of VGSCs is a means to induce V_{th} hyperpolarization in spinal motoneurons.

Given that veratridine is a Na^+ channel agonist, we also investigated whether changes in a TTX-sensitive I_{NaP} known to contribute to total PIC amplitude may also be mediating V_{th} hyperpolarization. The present results demonstrate that there is no relation between the veratridine induced hyperpolarization of V_{th} and the amplitude or $V_{1/2}$ of I_{NaP}. This is supported by three main findings: (1) V_{th} hyperpolarization can occur in the presence of a depolarizing shift of PIC $V_{1/2}$ (n = 9) (see Fig. 6B); (2) V_{th} hyperpolarization can occur in the presence of decreased PIC amplitude (n = 2); and (3)

V_{th} hyperpolarization occurs rapidly following veratridine administration while PIC amplitude facilitation takes between 5 and 10 minutes (see Fig. 5). Furthermore, the HEK cell data demonstrates that veratridine decreased the persistent Ca^{2+} current and had no effect on the voltage dependence for activation, implying that this current was not responsible for changes in PIC amplitude increases or changes in the activation kinetics in the whole cord preparation. This is again in agreement with a computer modeling study which suggested that I_{NaP} was unlikely to contribute to V_{th} hyperpolarization during locomotion in decerebrate cats. This finding is particularly interesting given the work has highlighted the importance of I_{NaP} in neuronal excitability. For example, I_{NaP} has been shown to play important roles in amplifying synaptic input (Alzheimer *et al.*, 1993a; Stuart & Sakmann, 1995; Schwindt & Crill, 1995) repetitive firing (Harvey *et al.*, 2006b; Lee & Heckman, 2001), fictive locomotion (Zhong *et al.*, 2007). Recent work has also demonstrated I_{NaP} as playing a vital role in action potential initiation during rhythmic firing and slowly rising inputs (Lee & Heckman, 2001; Kuo *et al.*, 2006). It cannot be concluded from these studies however that the I_{NaP} is necessary for spike initiation resulting from transient inputs and therefore may not be necessary to induce V_{th} hyperpolarization. In spinal motoneurons *in vivo* for example, Lee and Heckman (2001) concluded that although a reduction in the fast PIC mediated primarily by I_{NaP} eliminated rhythmic firing, cells were still capable of generating spikes mediated by I_{NaT}.

Dissociation of enhanced I_{NaP} and V_{th} hyperpolarization was also evident in transgenic mice that develop amyotropic lateral sclerosis-like symptoms. Kuo et al. (2004) tested the electrophysiological properties of cultured spinal motoneurons from these presymptomatic mice and demonstrated hyperexcitability as indicated by an

enhanced slope of the frequency/current relation and maximal firing rate. However V_{th} was not affected. A subsequent study by Kuo et al. (2006) in the same cell type demonstrated an enhanced I_{NaP} in high input conductance cells. Riluzole, presumably through a block of I_{NaP} prevented repetitive firing in response to slowly rising inputs while the ability of transient input to generate single spikes was unaffected. These experiments by Kuo and colleagues suggest that enhancing I_{NaP} can increase motoneuron excitability without a hyperpolarization of V_{th}. This is further support for our data, namely that an enhanced I_{NaP} does not necessarily result in V_{th} hyperpolarization.

On the other hand, Ostman et al. (2008) concluded that a GTP induced increase in I_{NaP} amplitude was necessary to induce V_{th} hyperpolarization in sensory neurons. The increased I_{NaP} amplitude in these neurons however was generated using the $Na_v1.9$ channel subtype (Ostman *et al.*, 2008), which is not present in spinal motoneurons (Catterall *et al.,* 2005). In neocortical slices, Astman et al. (1998) found that although activation of PKC induced a decrease in I_{NaP} amplitude, it also induced V_{th} hyperpolarization mediated by a negative shift in the voltage dependency for activation of the I_{NaP}. Thus, while enhancing the I_{NaP} through either an increased amplitude or a hyperpolarization of its' activation voltage can lead to V_{th} hyperpolarization, our data suggest that an enhanced I_{NaP} is not necessary to induce V_{th} hyperpolarization in spinal motoneurons. Differences between our findings and those of others may be due to the use of different neuronal populations expressing different types of VGSCs, each of which may have varying levels of importance for action potential initiation.

Currently it is unclear which Na^+ channel isoform generates the I_{NaP} and whether the Na^+ channel(s) subtype that generates the I_{NaP} and/or the I_{NaT} are the same or

different. It has been suggested that the channels generating the I_{NaT} and I_{NaP} may be of the same subtype(s) and that the I_{NaP} may represent a subpopulation of Na^+ channels that fail to inactivate (Alzheimer *et al.*, 1993b), have altered gating kinetics (Taddese & Bean, 2002). It is interesting to note that when $Na_v1.6$ channels are lacking the ß subunit the voltage dependence for their activation is depolarized compared to that of the $Na_v1.1$ or $Na_v1.2$. When expressing the ß subunits, all 3 isoforms have the same activation voltage. In spinal motoneurons it is generally thought that the Na^+ channels generating the I_{NaP} are activated at a membrane potential ~10 mV more negative than that of the NaT channels responsible for the action potential. Perhaps the I_{NaT} is generated to a large extent by $Na_v1.6$ channels lacking the expression of their ß subunits and that a subpopulation of $Na_v1.6$ channels expressing ß subunits provides the majority of current for the I_{NaP} with a correspondingly more hyperpolarized membrane potential.

Our data supports the conclusion that the population of Na^+ channels modified to induce V_{th} hyperpolarization are of the NaT population. If the channels generating the I_{NaP} were modified by veratridine to induce V_{th} hyperpolarization, this should have been evident via a hyperpolarization of their $V_{1/2}$, which was not the case. In addition, we do not know how many Na^+ channels are needed to be modulated in order to induce V_{th} hyperpolarization. It may be that a saturation effect occurs in that a relatively small number of Na^+ channels are needed to induce V_{th} hyperpolarization. The extra current through these channels may be sufficient to induce V_{th} hyperpolarization without increasing the I_{NaP} amplitude. This would explain why V_{th} hyperpolarization was seen within one minute of veratridine administration to the bathing solution in the *in vitro* preparation while enhanced I_{NaP} amplitude could take much longer. In addition, while

motoneuron I_{NaP} amplitude could increase substantially following several minutes of exposure to veratridine, the amount of V_{th} hyperpolarization did not change. Our conclusions that the subset of Na^+ channels generating the I_{NaT} were modified and induced a V_{th} hyperpolarization.

Putative mechanism(s) of V_{th} hyperpolarization

Motoneurons in the spinal cord are innervated densely by monoaminergic fibres originating within the brainstem which are known to have a potent effect on motoneuron excitability (for review see Rekling et al., 2000).The ability of monoamines (5-HT and NA) to induce V_{th} hyperpolarization in ventral horn neurons was demonstrated by Fedirchuk and Dai (2004) using the *in vitro* isolated spinal cords of neonatal rats. This was further supported by Gilmore and Fedirchuk (2004) who demonstrated that cooling of the cervical spinal cord or the addition of ketanserin (a 5-HT antagonist) to the bathing solution eliminated the V_{th} hyperpolarization seen during brainstem stimulation evoked locomotor-like activity. Increased excitability of neurons via these monoamines has been extensively studied and reported. Monoamines have been shown to induce depolarization of neurons (Connell & Wallis, 1988; Takahashi & Berger, 1990; Elliott *et al.*, 1999), reduce afterhyperpolarization amplitude (Madison & Nicoll, 1986), enhance membrane oscillatory behaviour, facilitate the expression of plateau potentials (Hounsgaard & Kiehn, 1989; Hounsgaard *et al.*, 1988) and enhance PICs (Perrier & Hounsgaard, 2003). The ionic mechanisms known to contribute to monoaminergic enhancement of neuronal excitability include: (1) inhibition of a fast inward rectifier current (I_{KIR}) and leak currents (Kjaerulff & Kiehn, 2001); (2) enhancement of a slow inward rectifier current carried by

K^+ and Na^+ ions (I_h) (Wang & Dun, 1990; Takahashi & Berger, 1990; Kjaerulff & Kiehn, 2001); and (3) facilitation of a low voltage-activated Ca^{2+} current (Berger & Takahashi, 1990). However the ionic mechanism(s) by which monoamines can produce a V_{th} hyperpolarization in spinal motoneurons remain unknown. Given that the identification of Na^+ channels as a site for neuromodulation has been shown (see Cantrell & Catterall, 2001) it is probable that the neuromodulators 5-HT and NA enhance motoneuron excitability through a modulation of VGSCs.

The monoamines are coupled to G-protein mediated intracellular pathways that when activated may induce a phosphorylation of VGSCs that effects their voltage dependency for activation and induces V_{th} hyperpolarization in spinal motoneurons. Modulation of VGSCs to alter cell excitability has been demonstrated in various cell types. In neostriatal cells, dopaminergic modulation of Na^+ channels via a PKA coupled pathway has been demonstrated with varying effects based on the receptor type activated. For example, D1 receptor activation inhibits peak Na^+ current and depolarizes V_{th} for action potential initiation while activation of D2 receptors enhances the peak Na^+ current and accelerates depolarization. Na^+ channel modulation has also been suggested to contribute to altered firing patterns in pyramidal cells resulting in depolarization of V_{th} and slower firing rates (see Cantrell & Catterall, 2001). Varying effects based on activation of the PKC pathway have also been reported. In rat hippocampal neurons, activation of muscarinic receptors reduced peak Na^+ current without affecting activation/inactivation kinetics of Na^+ channels (Cantrell *et al.*, 1996). In contrast, Franceschetti et al. (2000) demonstrated that activation of the PKC pathway produced a leftward shift in NaT channel activation and steady-state inactivation, as well as a

decrease in I_{NaT} peak amplitude. As mentioned, PKC pathway activation has also been demonstrated to induce V_{th} hyperpolarization (Astman *et al.*, 1998). Using a whole-cell slice patch clamp configuration, Dai et al. (2009) investigated whether the 5-HT induced V_{th} hyperpolarization was mediated via a PKC dependent pathway. PKC pathway activation in ventral horn neurons of neonatal rats decreased excitability via a reduced PIC amplitude, depolarized PIC onset, and a decreased I_{NaT} amplitude. Its effects on V_{th} were variable, causing either no change or a depolarization. 5-HT however induced a V_{th} hyperpolarization and was not altered by PKC activation or inactivation, suggesting the modulation of V_{th} could be mediated via multiple pathways (Dai *et al.*, 2009).

Conclusion

We used a Na^+ channel agonist to demonstrate that modulation of VGSCs can induce V_{th} hyperpolarization in spinal motoneurons. This effect is mediated through a hyperpolarization of the voltage needed to activate Na^+ channels mediating a I_{NaT} and does not depend on an enhancement of PICs.

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Figure Legends

Figure 1: Measurement of PIC amplitude and $V_{1/2}$

PICs are recorded by a slow voltage bi-ramp of 10 seconds duration from a holding potential of -70 mV to a peak voltage of +10 mV. The leak current is subtracted before measuring amplitude and $V_{1/2}$ for PIC. PIC $V_{1/2}$ is attained by fitting the PIC trace to a Boltzman charge-voltage equation (see equation 1):

$$\text{Equation 1. } F(V) = \frac{Imax}{1 + e^{(Vmid-V)/VC}} + C$$

V = membrane potential; Vmid = membrane potential at which current is half-maximal (defined as $V_{1/2}$ in the text); Vc = slope or the voltage required to change the current e fold; C = constant offset term.

Figure 2. Bath applied veratridine hyperpolarizes V_{th}

Prior to the application of veratridine, depolarizing steps that increased in +2 mV increments were applied to motoneurons and the resulting whole-cell current recorded. A: In control, a step to -32 mV from a holding potential of -60 mV was needed to elicit a fast inward current (indicated by arrow). B: After the administration of veratridine to the bath (30 μM), a step to -36 mV was sufficient to activate the fast inward current. This demonstrated a -4 mV hyperpolarization of V_{th}. In panels A and B, subthreshold and

suprathreshold voltage steps are shown (for further description see Results section). Recordings were made in the presence of synaptic blockers.

Figure 3. Veratridine hyperpolarized V_{th} in all motoneurons tested

Relative change in V_{th} of all motoneurons following the administration of 30 μM veratridine to the bath.

Figure 4. Veratridine increases PIC amplitude

Shows the slow voltage ramp (lower trace) and the corresponding current record (upper traces, leak subtracted) used to test for PICs. PICs have been facilitated by the blockade of K+ channels using TEA (10 mM) and 4-AP (5mM). Note the PIC amplitude (40.7 pA) prior to and following the administration of veratridine (180.6 pA).

Figure 5. Veratridine effects on V_{th} and PIC amplitude have different time courses

Minutes 2-6: 30 μM veratridine causes V_{th} hyperpolarization (blue points) and a decreased PIC amplitude (red points). Minutes 9-15: PIC amplitude increases while V_{th} remains relatively unchanged. The dashed line represents the normalized control value for PIC amplitude and V_{th}. Example is data recorded from a single motoneuron.

Figure 6: Veratridine facilitates PIC amplitude but has variable effects on PIC $V_{1/2}$

A1: Shows a slow voltage ramp (lower trace) and the corresponding current records (upper traces; black = control; red = veratridine) used to test for PICs in the same motoneuron. The boxed area is expanded in A2. A2: PIC $V_{1/2}$ for control and veratridine

are indicated by arrows of the same colour (-39.8 mV and -27.3 mV, respectively). B1 shows same as A1 in different motoneuron. B2 shows PIC $V_{1/2}$ for control and veratridine indicated by arrows of the same colour (-38.4 mV and -42.1 mV, respectively). PICs have been facilitated by the blockade of K+ channels using TEA (10 mM) and 4-AP (5mM). V_{th} was hyperpolarized using step depolarizations following veratridine in A (4 mV) and B (5 mV).

Figure 7. Veratridine increases PIC amplitude in the presence of calcium channel blockers

A: Shows a voltage ramp (lower trace) and the corresponding current traces (upper traces: control = black; Cd^{2+} (500 μM) = blue; veratridine (30 μM) = red) from a motoneuron. The boxed area is expanded in B. Reduction in PIC amplitude following Cd^{2+} administration occurred within 5 minutes. By 5 minutes after veratridine administration, PIC amplitude began to recover, approaching the control amplitude.

Figure 8. Veratridine reduces total I_{NaT} but increases PIC in cells expressing recombinant $Na_v1.2$ sodium channels

A: Peak current-voltage relationships recorded under control conditions, in the presence of 30 μM veratridine and after switching the superfusate back to control solution (washout). Currents were elicited with 10 ms step depolarizations at 3 s intervals (n = 4 cells / curve; error bars are +/- SD). B: Veratridine modification of sodium channels creates a persistent component of the current. In the presence of 30 μM veratridine, a depolarizing voltage step elicits both a persistent current component (after the initial fast

peak) and a prolonged deactivation (tail-current) upon repolarization of the membrane potential. Currents were elicited with 15 ms voltage steps to -10 mV from a holding potential of -110 mV. Control and test recordings were made 7 minutes apart while the washout recordings were made 9 minutes after the control recording. C: Veratridine modified channels produce large inward currents in response to slow ramp depolarizations. Ramp currents were elicited with 16 mV/s voltage ramps demonstrated a concentration-dependent increase in amplitude that was reversible upon washout of the toxin. D: The voltage-dependence channel activation is shifted in the hyperpolarizing direction in the presence of veratridine. Control conductance-voltage curve was generated from peak current amplitude elicited by 10 ms step depolarizations from a holding potential of -110 mV. The conductance-voltage curve in the presence of veratridine was generated from tail current measurements so as to reflect a pure population of modified channels. Data was fitted using a Boltzmann equation of the form; G/Gmax = minimum + (maximum - minimum) / (1 + exp ((V $V_{1/2}$ - X) / slope); G is the conductance, maximum was constrained to 1. Values for control are; V $V_{1/2}$ = -29 mV, slope = 5.4. In the presence of veratridine the values are; V $V_{1/2}$ = -41 mV, slope = 3.6. n = 4 cells / curve, error bars are +/- SD.

Figure 1

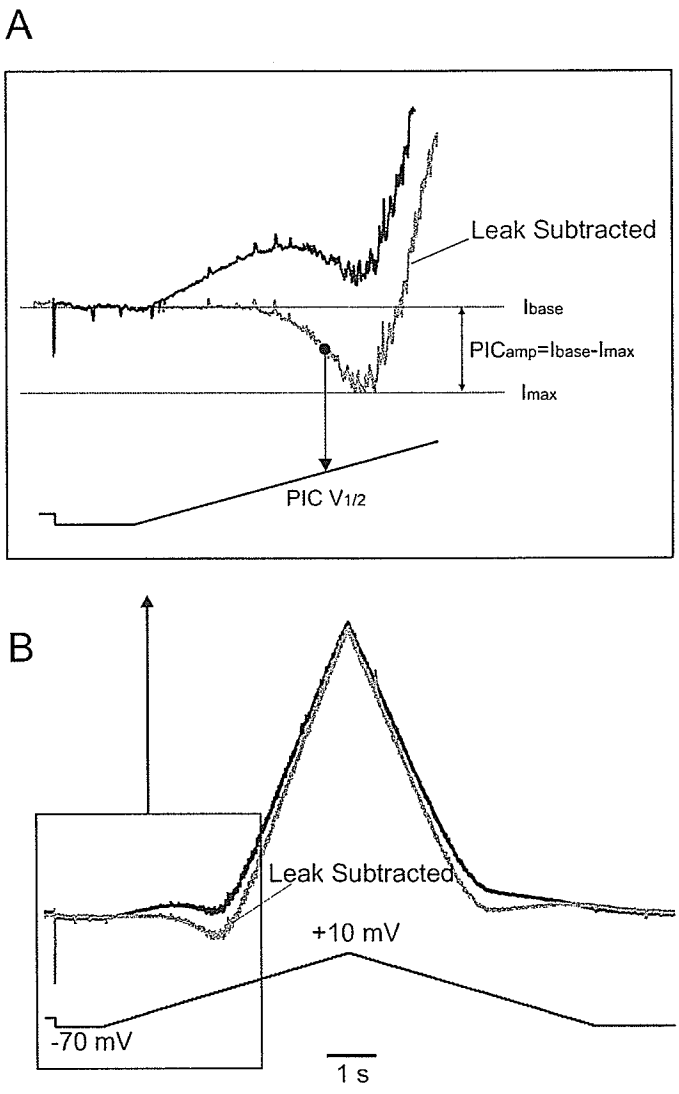

A
Leak Subtracted
Ibase
PICamp=Ibase-Imax
Imax
PIC V1/2
B
Leak Subtracted
+10 mV
-70 mV
1 s

Figure 2

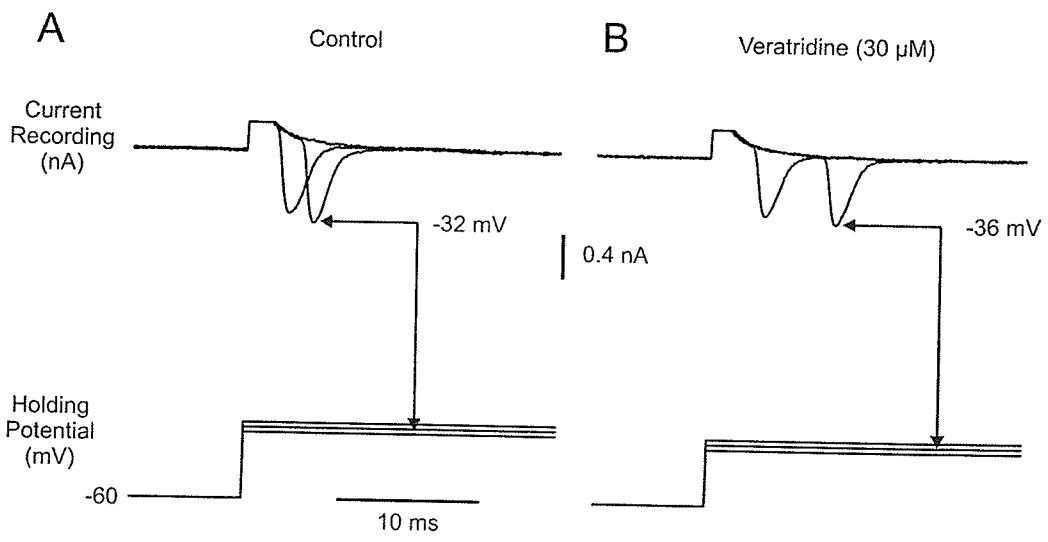

A
Control
B
Veratridine (30 μM)
Current
Recording
(nA)
-32 mV
0.4 nA
-36 mV
Holding
Potential
(mV)
-60
10 ms

Figure 3

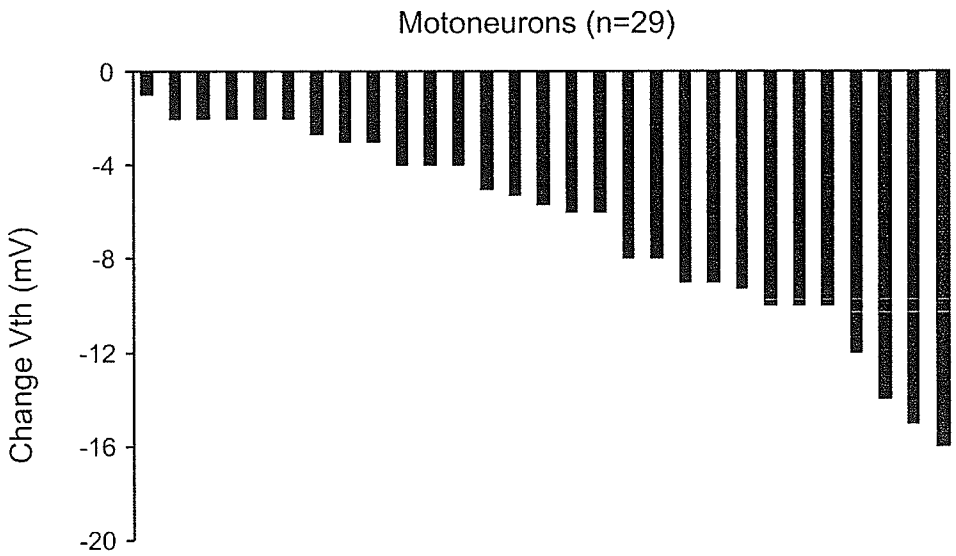


Figure 4

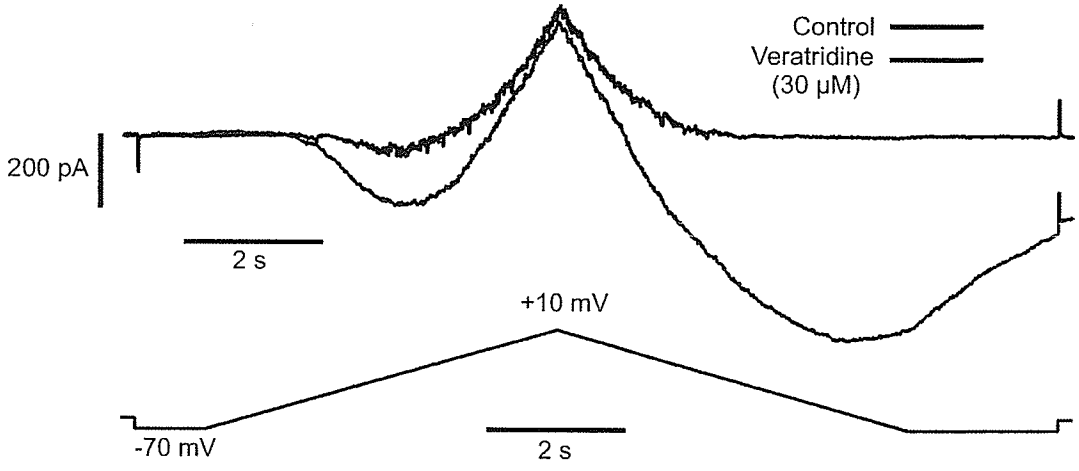


Figure 5

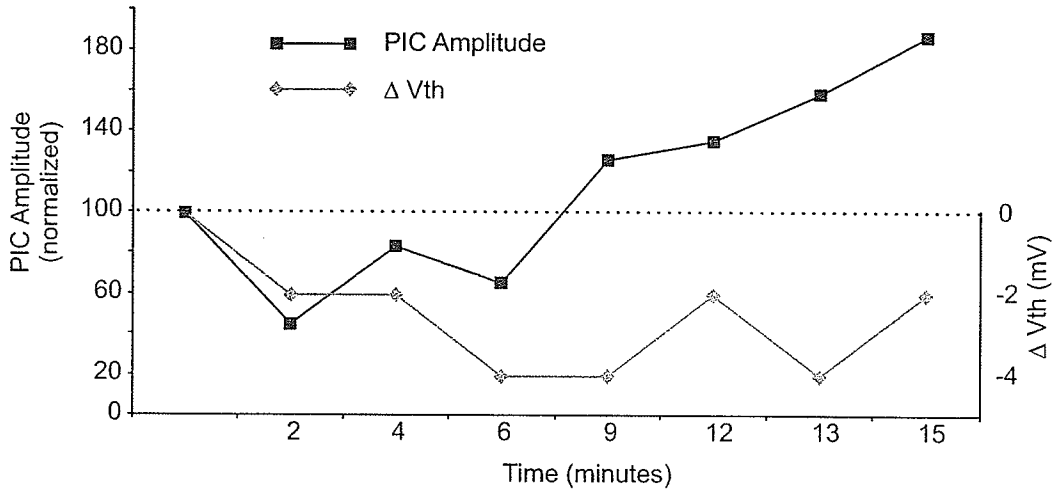

PIC Amplitude
Δ Vth
PIC Amplitude (normalized)
180
140
100
60
20
0
0
-2
-4
Δ Vth (mV)
2
4
6
9
12
13
15
Time (minutes)

Figure 6

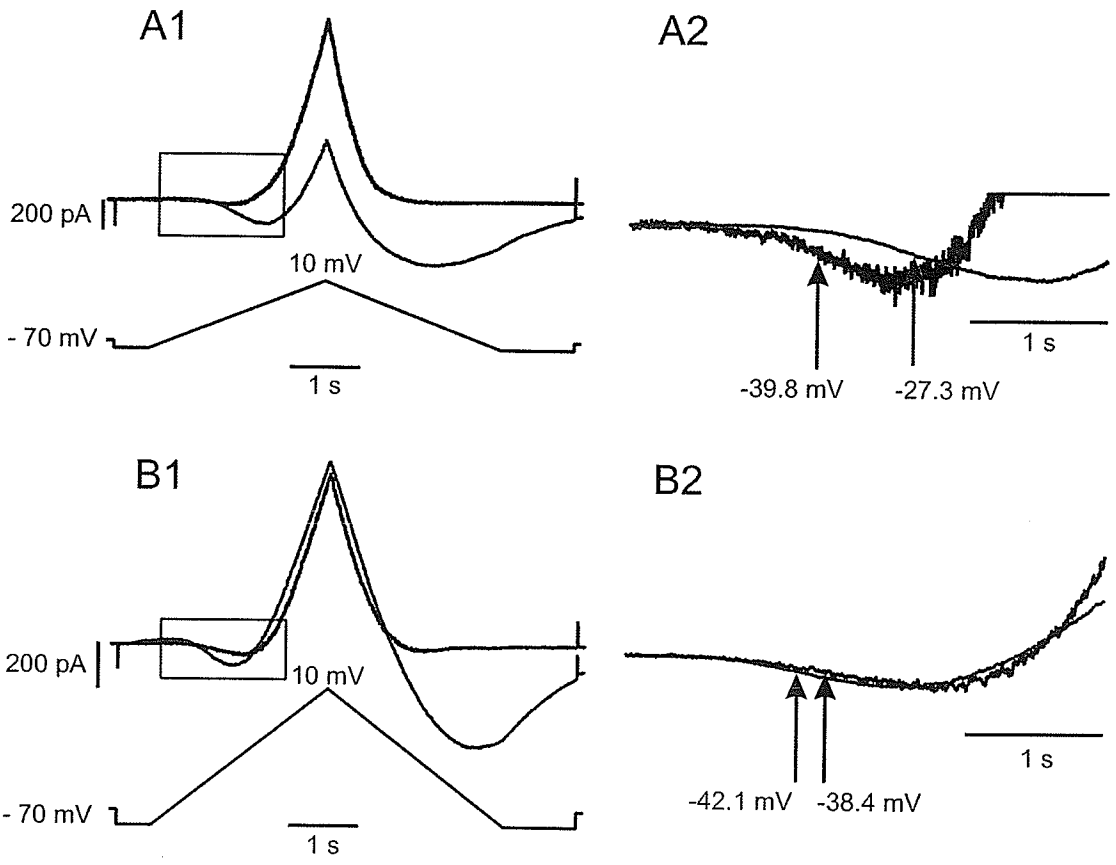

A1
200 pA
10 mV
- 70 mV
1 s
A2
1 s
-39.8 mV
-27.3 mV
B1
200 pA
10 mV
- 70 mV
1 s
B2
1 s
-42.1 mV
-38.4 mV

Figure 7

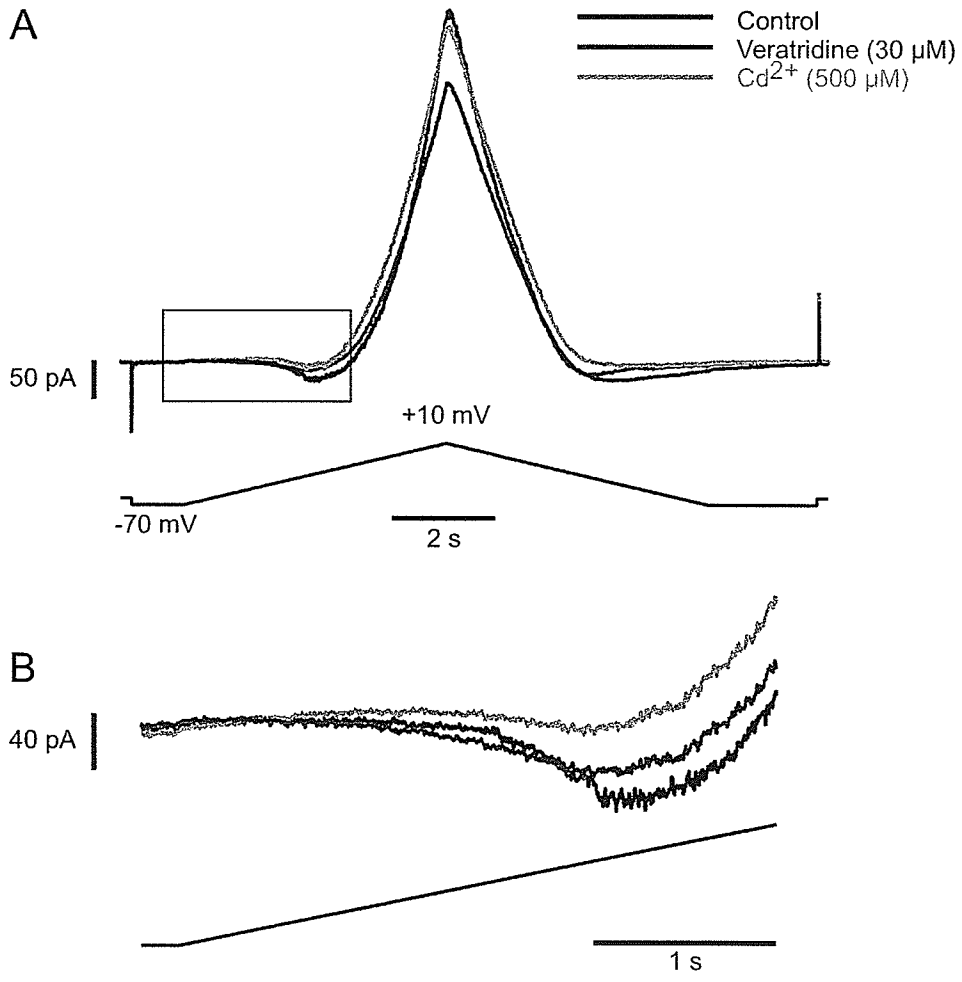
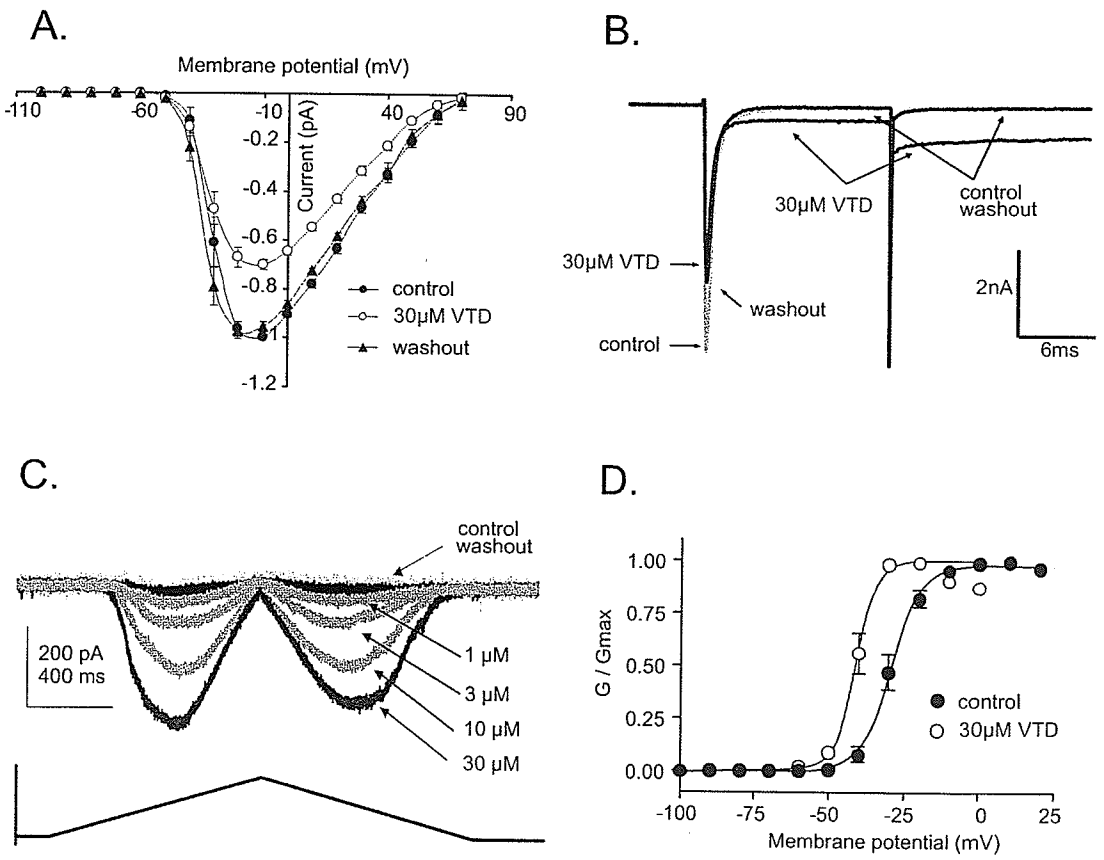

A
Control
Veratridine (30 μM)
Cd2+ (500 μM)
50 pA
+10 mV
-70 mV
2 s
B
40 pA
1 s

Figure 8


A.
Membrane potential (mV)
-110
-60
-10
40
90
-0.2
-0.4
-0.6
-0.8
-1
-1.2
Current (pA)
control
30μM VTD
washout
B.
30μM VTD
control
washout
30μM VTD
washout
2nA
6ms
C.
control
washout
200 pA
400 ms
1 μM
3 μM
10 μM
30 μM
D.
1.00
0.75
0.50
0.25
0.00
G / Gmax
-100
-75
-50
-25
0
25
Membrane potential (mV)
control
30μM VTD

III. Paper 2: Motoneuron excitability is enhanced during fictive scratch by voltage threshold hyperpolarization and reduced afterhyperpolarization amplitude

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Running Head: V_{th} hyperpolarization during fictive scratch

Keywords: voltage threshold, motoneuron, spinalization, conductance, decerebrate cat

Abstract

Hindlimb motoneuron firing is facilitated at the onset of brainstem stimulation evoked fictive locomotion in the decerebrate cat by a lowering of the voltage threshold (V_{th}) for action potential initiation and a reduction of the post-spike afterhyperpolarization (AHP). Whether these state-dependent changes in motoneuron excitability occur during motor behaviours not involving brainstem stimulation is not known. In the present study fictive scratch was produced in the decerebrate cats following topical application of curare to the cervical spinal cord and manual stimulation of the ipsilateral pinna. Measurements of the V_{th} and AHP of spikes evoked by intracellular injection of depolarizing ramp currents or rectangular wave pulses were compared during control and fictive scratch. In 26/31 antidromically identified motoneurons in intact cats. V_{th} became hyperpolarized (mean hyperpolarization -6.3 ± 4.4 mV; range -1.1 to -17.2 mV) with the onset of fictive scratch. In two cats fictive scratch was induced after complete transection of the spinal cord at C1. The V_{th} of 12 of 13 motoneurons recorded in these preparations was hyperpolarized (mean hyperpolarization: -7.1 ± 1.4 mV mV; range: -1.3 to -26.2 mV) during fictive scratch. In addition to changes in V_{th}, there was a robust reduction of AHP amplitude during both the approach (tonic flexion) and rhythmic phases of fictive scratch in both spinal intact and spinal transected preparations. V_{th} hyperpolarization and AHP amplitude reduction recovered soon after the cessation of an episode of fictive scratch. AHP amplitude reduction was not associated with a large increase in motoneuron conductance. Motoneuron excitability is enhanced during fictive scratch through a hyperpolarization of the V_{th} for action potential initiation and a reduction in AHP amplitude. The persistence of these effects in spinal

transected animals suggests the existence of a previously unrecognized intraspinal mechanism for increasing motoneuron excitability.

Introduction

The onset of centrally generated motoneuron activity induces changes in the electrical properties of spinal motoneurons that enhance their excitability and facilitate firing. During fictive locomotion in the decerebrate cat, motoneurons exhibit decreased afterhyperpolarization (AHP) amplitude (Brownstone *et al.*, 1992), the emergence of voltage-dependent depolarizing currents (Brownstone *et al.*, 1994) and decreased rheobase currents (Krawitz *et al.*, 2001). In addition, motoneuron excitability during fictive locomotion is enhanced by a hyperpolarization of the V_{th} for action potential initiation. This locomotor-dependent V_{th} hyperpolarization occurred in every motoneuron examined (mean change of -8 mV) and recovered within seconds to minutes of locomotor cessation. This study provided the first evidence of a rapidly induced, state-dependent hyperpolarization of the V_{th} as a novel mechanism utilized by the motor system to facilitate recruitment and firing of spinal motoneurons. Further work by Gilmore and Fedirchuk (2004) in the neonatal rat showed that descending serotonergic pathways could be one source of V_{th} hyperpolarization in spinal motoneurons and interneurons. The ability of the monoamines serotonin (5-HT) and noradrenaline (NA) to induce V_{th} hyperpolarization in ventral horn neurons was confirmed by Fedirchuk and Dai (2004). A computer modeling study based on the locomotor data in decerebrate cats suggested that the most effective means to induce V_{th} hyperpolarization was through modulation of the fast Na^+ current underlying action potentials. By increasing the Na^+ channel conductance by 50% or by hyperpolarizing its voltage-dependency for activation by 3 mV in the initial segment V_{th} changes were produced that closely mimicked those seen *in vivo* (Dai *et al.*, 2002). Thus, it is possible that monoamines induce V_{th} hyperpolarization by modulating

the voltage-dependency for activation of Na^+ channels.

It remains unclear whether V_{th} hyperpolarization occurs during motor behaviours other than locomotion and whether it is dependent upon the supraspinal activation of pathways by the electrical brainstem stimulation used to produce locomotion. To answer these questions, we induced fictive scratch in an adult cat preparation. Scratch is a cyclical hindlimb motor behaviour different from locomotion (Sherrington, 1906, 1910, and 1917). It is an automatic response to cutaneous stimuli around the ear, head, or neck consisting of rapid rhythmic movements of the ipsilateral hindlimb in order to remove the stimuli. The advantage of using the adult cat preparation is the ability to generate scratch following isolation of the spinal cord from descending input, including that from the brainstem after spinal transection (Sherrington, 1903). Thus, the goals of the present study were to use fictive scratching in both the spinal intact and spinal transected decerebrate cat to determine whether a V_{th} hyperpolarization of lumbar motoneurons: (1) occurs during fictive scratch, (2) requires electrical brainstem stimulation, and (3) requires an intact spinal cord.

Methods

Surgical procedures

Data were obtained from 9 cats of either sex. All surgical and experimental protocols were in compliance with the guidelines set out by the Canadian Council for Animal Care and the University of Manitoba. Anaesthesia was induced and maintained with halothane (1-2%) delivered in an oxygen/nitrous oxide mixture (30%/70%). Administration of the anesthetic mixture was maintained by a tracheotomy tube. The level of anaesthesia was monitored by confirming the absence of pedal withdrawal

reflexes periodically and by continuously monitoring arterial blood pressure and muscle tone. Canulas were inserted in the left femoral and the right jugular veins for drug administration. Atropine (0.05 mg/kg subcutaneous), saline (10 ml subcutaneous), and dexamethasone (2 mg/kg intravenous) were given at the beginning of the surgery. A buffer solution (5% glucose, 0.84% bicarbonate solution; 5 ml/h) was continuously infused through the jugular vein for blood pH maintenance. Blood pressure was monitored from the right carotid artery using a transducer. The CO_2 levels and respiratory rhythm were monitored by a sensor inserted into the tracheotomy tube. The bladder was catheterized through the urethra.

The peripheral nerves of the left hindlimb innervating the following muscles were dissected and mounted for recording and stimulation: posterior biceps and semitendinosus, PbSt; semimembranosus and anterior biceps, SmAb; lateral gastrocnemius and soleus, LGS; medial gastrocnemius, MG; plantaris, Plant; tibialis anterior, TA; extensor digitorum longus, EDL; peroneus longus, PLong; flexor digitorum and hallucis longus, FDHL; common peroneal, CP; tibial, Tib; sartorius, Sart. Nerve recordings from the right (contralateral) hindlimb included: TA, Sart, and MG (the remaining nerves were cut). The adductor tendons of both hips were cut.

Following a dorsal laminectomy exposing segments L4 to L7 of the spinal cord, the cat was transferred to a sterotaxic recording frame. The dorsal aspect of the cervical spinal cord was exposed at C1-C2 for topical application of curare to elicit fictive scratch (see below). Mineral oil pools were made for the spinal cord and both hindlimbs, and the dissected nerves were placed on conventional silver hook bipolar electrodes for stimulation and recording. The temperature of the animal was maintained by a heating

pad and radiant heat lamps. After mechanical removal of the cortex, a blunt transection of the brainstem was performed at the precollicular, postmamillary level. All tissue rostral to the transection was removed. After the decerebration, anaesthesia was discontinued and the animal was paralyzed (pancuronium bromide, 0.1 mg/kg supplemented every 45 minutes) and artificially ventilated. Bilateral openings in the chest wall were used to minimize respiratory movements. Decreases in blood pressure were countered by the intravenous administration of a blood volume expander (dextran).

Fictive scratch

According to Kuhta and Smith (1990), the scratch reflex in intact adult cats consists of three phases, approach, cyclic, and return. During the approach phase there is an initial period of tonic flexion in the ipsilateral hindlimb as it is positioned toward the head combined with simultaneous extension of the contralateral hindlimb. This is followed by the cyclic or rhythmic phase characterized by strict and rapid alternation of flexors and extensors of the ankle, knee, and hip joints resulting in repetitive scratching of the irritated area of the skin. During the return phase the hindlimb is returned to the pre-response posture. The efferent activity during the fictive scratch reflex (fictive due to immobilization of the limb) is similar to that of real scratch with a cycle duration of ~250 ms (i.e. flexor phase ~200 ms, extensor phase ~50 ms) (Berkinblit *et al.*, 1978).

Fictive scratching was elicited in the left hindlimb by first placing a small piece of curare-soaked cotton (0.1 – 0.3%) on the left C1-C2 dorsal roots (Deliagina *et al.*, 1975; Berkinblit *et al.*, 1978) followed by mechanical stimulation of the left pinna or left side of the face. Hindlimb ENG recordings were filtered (30 Hz to 3 kHz), rectified and

integrated before digitization at 500 Hz. All signals were captured and analyzed using software developed within the Spinal Cord Research Centre and running on a PC in the Linux operating system. Runs of fictive scratch were typically captured in 1 to 3 minute segments.

Intracellular recordings

The objective of this study was to compare the membrane potential at which action potentials were initiated (V_{th}) during control and fictive scratch conditions. Intracellular recordings (digitized at 10 kHz) were made from anitdromically identified lumbar motoneurons using 2M potassium citrate or 2M sodium citrate filled glass electrodes (tip size 1.6 – 1.9 mm). Use of the discontinuous current clamp (DCC) mode of an Axoclamp 2A amplifier (Axon instruments) permitted reliable measurements of membrane potential during injection of large intracellular currents. The ability of the electrode to pass the current without rectification was continuously assessed using a high speed, high gain oscilloscope trace of the electrode voltage. Under control conditions without fictive scratch, injection of a ramp or pulses of depolarizing current was used to evoke an action potential(s) in the motoneuron. Fictive scratch was initiated later in the same trial, and the intracellular current injection was repeated. The extracellular DC potential recorded immediately after withdrawing the microelectrode was measured and subtracted from the intracellular potential. Recordings in which the intracellular DC values were suspected of drifting were discarded.

Voltage threshold measurement

Voltage threshold was measured for the first spike elicited either by a depolarizing ramp or more commonly, by depolarizing rectangular pulses of intracellularly injected depolarizing current. Measurements are reported only for the first spike evoked to avoid the possibility that previous spikes may influence the subsequent V_{th} by either spike accommodation or inter-spike membrane potential trajectory. V_{th} was defined as the membrane potential at which depolarization increased at $\geq 10\ Vs^{-1}$. At the 10 kHz sampling rate used, the reported V_{th} value was the membrane potential of the data point that was ≥ 1 mV more depolarized than the preceding point. This measure was chosen because it corresponded well to the distinct rising portion of the action potential and was easily selected by independent observers. The same definition of V_{th} was employed by Krawitz et al. (2001). Measurements in which there was a change in V_{th} of $\geq$1 mV in either the depolarizing or hyperpolarizing direction were defined as significant. All V_{th} values reported for both control and fictive scratch conditions were from an average of 3-4 spikes. The V_{th} for each motoneuron was measured in the same data file during control conditions and fictive scratch. Each cell thus served as its own control. In two cats an acute and complete transection of the spinal cord at C1 was made to eliminate descending input to the spinal cord. in both preparations V_{th} was recorded in some motoneurons before spinal transection. In these cells, V_{th} measurements were made in the same manner as previously described.

Afterhyperpolarization assessment

Two methods of evoking action potentials were used to compare control and fictive scratch AHP amplitudes: (1) ramp current injections and (2) suprathreshold 0.5 ms current pulses. The intensity of the injected currents was above threshold for eliciting action potentials and varied from cell to cell. By eliciting spikes throughout the fictive scratch cycle it was possible to determine if changes in AHPs that may have been related to the phase of the scratch cycle in which the spike occurred. A quantitative measure of AHP amplitude could not be made due to the lack of a good baseline from which to measure the peak amplitude of the AHP. Brownstone et al. (1992) used the V_{th} as baseline when measuring the AHP during fictive locomotion; however, we now know that this underestimates the AHP because fictive locomotion induces V_{th} hyperpolarization (for discussion of difficulties in measuring AHP amplitude see Results section).

Conductance measurements

In one cat, the averaged peak voltage deflection resulting from a 5 nA, 4-5 ms hyperpolarizing current pulse was used to assess changes in input resistance (R_{in}) between control and fictive scratch conditions. Pulses occurring immediately following a spike were not averaged to avoid potential contamination with the activation of voltage-gated conductances. The estimated conductance was then obtained by dividing the amplitude of the current injected during the pulse by the ensuing voltage deflection.

Results

V_{th} during fictive scratch was assessed in 44 antidromically identified motoneurons innervating a variety of hindlimb muscles of 9 adult decerebrate cats. Only motoneurons with action potential amplitudes ≥ 60 mV during control conditions elicited by depolarizing current pulses or antidromic stimulation were included in the analysis. Any cells with obvious changes in action potential shape, amplitude, or duration were excluded from the set of 44 motoneurons reported here. V_{th} was determined prior to the initiation of fictive scratch using depolarizing current ramps and/or pulses and was compared to the V_{th} of the same motoneuron during different phases of the fictive scratch cycle. The large range of rheobase values (2-32 nA) suggests that motoneurons innervating both slow (low rheobase) and fast (high rheobase) twitch muscle fibres were included in the sample (Krawitz *et al.,* 2001). The sample of motoneurons included both flexors and extensors (4 flexors, 10 bifunctionals, and 30 extensors).

In 9 of 10 motoneurons examined, the V_{th} for action potentials evoked by current ramps was more depolarized (mean: 3.8 mV) than V_{th} obtained using a current pulse (Wilcoxon signed rank test; $P = 0.04$). This was likely due to accommodation of voltage-gated sodium channels leading to a more depolarized V_{th} during the depolarizing ramps. In these cells V_{th} values obtained using the current pulse were compared during scratch and control conditions. All comparisons of V_{th} during control and fictive scratch were made using the same technique.

V_{th} during fictive scratch was measured during: (1) motoneuron firing during the depolarizing phase of the SDP without intracellular current injection, (2) ramp and/or pulse current injection during the rhythmic phase of fictive scratch (alternating activity

between flexor and extensor ENG activity), (3) ramp current injection during the approach phase, and (4) current pulses delivered during the inhibitory phase of the SDP. In total (spinal intact and spinalized preparations), the V_{th} of 38/44 motoneurons (86.4%) became hyperpolarized during fictive scratch, while 4/44 (9.1%) became depolarized, and 2/44 (4.5%) showed no change. In all cells in which V_{th} was hyperpolarized during fictive scratch, the threshold of the first action potential evoked was hyperpolarized. Thus as in fictive locomotion, V_{th} hyperpolarization was not a consequence of previous action potentials. The following sections provide a more detailed description of V_{th} changes that occurred during fictive scratch.

There were no correlations between the amount of V_{th} hyperpolarization during fictive scratch and membrane potential ($r = 0.03$, $P = 0.90$, rheobase ($r = -0.07$, $P = 0.80$), or scratch drive potential (SDP) amplitude ($r = -0.38$, $P = 0.20$).

Spinal Intact

V_{th} hyperpolarization does not require electrical stimulation of the brainstem

Figure 1 shows ~18 s from a 3 minute data run in which action potentials in a GS motoneuron were first evoked (3rd trace from top) under control conditions by ramp-shaped intracellular current injection (lower trace). In this motoneuron the first action potential occurred when the current ramp reached ~ 6 nA. Panel B shown at an expanded time scale shows the V_{th} as measured using the criteria of a 10 Vs^{-1} depolarizing change in the membrane potential. Approximately 4 s later light manipulation of the ipsilateral pinna produced rapid alternation of extensor and flexor ENG activity characteristic of fictive scratch (1A upper traces). Panel C shows that the V_{th} was hyperpolarized during fictive scratch; in this case by 7.5 mV. This change in V_{th} during fictive scratch was not

accompanied by an obvious change in action potential duration. The first spike during fictive scratch is larger than control but has a similar peak depolarization. The V_{th} for successive action potentials on the same SDP is depolarized when compared to the first action potential, presumably due to accommodation (note also decreased overshoot).

In spinal intact cats, 26 of 31 cells demonstrated V_{th} hyperpolarization (mean hyperpolarization -6.3 ± 4.4 mV; range -1.1 to -17.2 mV) during fictive scratch.

Data were obtained from 27 cells in spinal intact decerebrate cats in which V_{th} was compared between spikes evoked by current injection (control) and spontaneous spikes in the depolarizing phase of the SDPs during fictive scratch. Another 4 cells did not fire spontaneously during fictive scratch. Control values were determined from current ramps for 10/27 cells (see Figure 1) and from current pulses for 17/27 cells. When control V_{th} values measured during intracellular current ramp were compared to the V_{th} of spikes firing spontaneously off of their respective SDPs, 10 of 10 cells demonstrated V_{th} hyperpolarization (mean hyperpolarization: -7.4 mV; range: -2.5 to -17.2 mV).

When control V_{th} values were recorded using intracellular current pulse injections, however, only 8 of 17 demonstrated a hyperpolarization of their V_{th} (mean hyperpolarization: -7.2 mV; range -2.0 to -16 mV). Four of the remaining 9 cells demonstrated V_{th} hyperpolarization during different phases of the fictive scratch cycle. Of the 4 cells that did not fire spontaneously off of their SDP, all 4 demonstrated V_{th} hyperpolarization (mean hyperpolarization: -5.2 ± 2.8 mV; range: -2.9 to -8.6 mV) when the V_{th} from firing produced by intracellular current injection during fictive scratch was compared to control.

V_{th} hyperpolarization occurs throughout the fictive scratch cycle

Fictive scratch consists of 3 changes in motoneuron membrane potential. The first occurs during the approach phase when extensor motoneurons are toncially hyperpolarized and flexor motoneurons tonically depolarized. The subsequent changes are the rapid depolarization and hyperpolarization of the motoneuron produced by the scratch central pattern generator (CPG). Figure 2A (top panel) shows a prominent approach phase, corresponding to the prolonged tonic activity in the TA ENG, prior to rhythmic alternation of flexor (TA) and extensor (GS) ENGs during fictive scratch. The shaded regions Figure 2A are shown on expanded time bases in panels 2B and 2C. In this example, an intracellular recording from a GS motoneuron was made and two identical ramp current injections were delivered. The first current injection was delivered prior to the onset of fictive scratch (control). It evoked an action potential as the current reached 14.6 nA with a V_{th} of -36.5 mV (Figure 2B). The second current injection was delivered just prior to and continuing throughout the majority of the approach phase of the fictive scratch cycle and evoked an action potential as the current reached 9.8 nA with a V_{th} of -43.9 mV (Figure 2C), representing a hyperpolarization of the V_{th} by 7.4 mV compared to control. Using this recording paradigm, 4 of 5 cells demonstrated a hyperpolarization of their V_{th} (mean hyperpolarization: -4.1 mV; range: -1.8 to -7.4 mV). One cell showed no change in V_{th} during the approach phase, but demonstrated a V_{th} hyperpolarization during the depolarized phase of the SDP. In 1 other motoneuron, intracellular current injection just prior to the onset of the approach phase of fictive scratch elicited an action potential with a hyperpolarized V_{th} (data not shown).

Figures 2B and 2C, show a reduction of 4.8 nA in the amount of intracellular current injection required to elicit action potentials at the onset of the approach phase. Increased motoneuron excitability represented as a decrease in the current needed to elicit an action potential however is difficult to quantify, since during each phase of the fictive scratch cycle, ionic conductances are activated by synaptic input to the motoneurons generated by the scratch CPG that will either decrease or increase the amount of current injection required to evoke action potentials. It is interesting to note however that when comparing the rheobase values generated by ramp current injections in both control and fictive scratch conditions, rheobase is decreased on average by 54% (i.e. 7.2 nA). This suggests that the CPG is generating large amounts of excitatory input to the motoneuron pool during fictive scratch.

Further evidence of V_{th} hyperpolarization throughout the fictive scratch cycle is illustrated in Figure 3. The middle trace in Figure 3A shows an intracellular recording of a GS motoneuron during fictive scratch. Random delivery of current pulses at approximately 2 Hz during fictive scratch allowed assessment of V_{th} during rhythmic depolarizations and hyperpolarizations of the motoneuron. Injection of a 19.4 nA current pulse prior to fictive scratch (control) produced an action potential with a V_{th} of -39.4 mV (Figure 3B). During the inhibitory phase when GS motoneurons are hyperpolarized and do not fire action potentials, a current pulse of 27.8 nA elicited an action potential with a V_{th} of -41.5 mV, taht was hyperpolarized by 2.1 mV compared to control. Motoneuron hyperpolarization during the flexor phase increased the current required to generate an action potential. As a result, current amplitude was manually adjusted during the bout of fictive scratch until firing was initiated. Using this paradigm 7 of 7 cells demonstrated a

hyperpolarized V_{th} during the inhibitory phase compared to control (mean hyperpolarization: -4.2 mV; range: -1.1 to -8.6 mV). V_{th} hyperpolarization did not persist following the cessation of fictive scratch. Recovery of V_{th} to within 2 mV of the control value typically occurred within 5 - 10 s of the end of ENG activity.

Spinal Cord Transected

V_{th} hyperpolarization does not require an intact spinal cord

Scratch can be elicited following spinal transection at C1 (Deliagina *et al.*, 1981). In order to determine whether changes in V_{th} occur without descending input to the cord, a complete transection of the spinal cord at the C1 segment was made in 2 cats. Figure 4B illustrates the fictive scratch pattern elicited following spinal transection (data taken from same experiment producing the fictive scratch pattern in Figure 4A, prior to spinalization). Note that the ENG activity patterns are similar during fictive scratch before and after acute C1 spinalization, with rhythmic alternation between the flexors (red) and extensors (blue).

Data were obtained from 13 cells in spinal cord transected decerebrate cats, comparing V_{th} from current-pulse evoked firing (control) to V_{th} of action potential evoked by fictive scratch SDPs (7 cells) or V_{th} of current-pulse evoked action potentials in 6 cells that were not recruited by the SDPs. Hyperpolarization of the V_{th} occurred in 12 of 13 cells (mean hyperpolarization: -7.1 mV; range: -1.3 to -26.2 mV); 1 cell showed no change. As was the case in the spinal intact preparations, there were no obvious changes in action potential shape or duration, nor were there any correlations between the amount of V_{th} hyperpolarization seen during fictive scratch with membrane potential ($r = -0.22$, $P = 0.68$), rheobase ($r = -0.12$, $P = 0.82$), or SDP amplitude ($r = 0.25$, $P = 0.69$).

Figure 5B and C shows an intracellular recording of a MG motoneuron prior to (control) and during fictive scratch in a preparation with a C1 spinal cord transection. Figure 5B shows that the V_{th} of an action potential elicited with a current pulse is -48.6 mV. During fictive scratch, rhythmic depolarizations induced cell firing with a V_{th} of -53.3 mV (Figure 5C). This represents a V_{th} hyperpolarization of 4.7 mV.

AHP amplitude is reduced during fictive scratch in the decerebrate cat

Figure 6A illustrates the same GS motoneuron recording as in Figure 2. Ramp current evoked firing in control and spontaneous firing fictive scratch are compared. The bars below the voltage traces indicate the spikes from which the AHP average was obtained. There is an obvious reduction in AHP amplitude following spikes during current-evoked firing in the approach phase when compared to spike AHP during current-evoked firing in control. Figures 6B and C are expanded views of the averaged AHPs for control and during fictive scratch. The decrease in AHP amplitude is evident following the spike in Figure 6D which shows the averaged spikes overlaid and aligned to the inflections at AHP onset (arrow). Similarly, the inset shows that when the V_{ths} are used to align the traces, there is an obvious reduction in AHP amplitude.

Although AHP amplitude is clearly reduced during fictive scratch, the lack of a good reference point from which to measure AHP amplitude makes a quantitative comparison between control and fictive scratch AHPs problematic. A previous study used V_{th} as a reference point from which to measure AHP amplitude during control and fictive locomotion (Brownstone *et al.*, 1992) as is illustrated in the inset. This technique however underestimates AHP amplitude because of the hyperpolarization of the V_{th}.

Using the membrane potential prior to the spike as the baseline is also problematic because the spikes that occur during fictive scratch are during the depolarizing portion of the SDP, thus masking the AHP amplitude due to the activation of excitatory conductances. High firing rates during fictive scratch and rapid changes in membrane potential produced by the SDP preclude the use of longer averages that would include the whole AHP duration (Figure 6D). It is clear, however, that there is a substantial reduction in the AHP during fictive scratch. Furthermore, this reduction also occurs during scratch in acute spinal preparations

AHP amplitude was reduced or absent in 34/34 motoneurons during spontaneous firing on the depolarized phase of the SDP. Notwithstanding the aforementioned limitations regarding AHP amplitude measurements, in 24 motoneurons a comparison of control (ramp or short pulse current threshold) AHP amplitude and AHP amplitude during the depolarized phase of the SDP was made. AHP amplitude reduction was evident when the AHP's were superimposed with V_{ths} aligned. Cells with a visual reduction in AHP amplitude during fictive scratch, defined as a membrane potential during the AHP that was more depolarized than that during control, were given a value of -1. Not surprisingly, there was a significant difference between AHP amplitudes during control *versus* scratch when compared using a Wilcoxon signed rank test ($P < 0.001$). Reduction of the AHP amplitude was evident throughout the fictive scratch cycle in both spinal intact and spinal transected preparations.

AHP amplitude reduction is not due to a high conductance state during fictive scratch

A recent report found that motoneurons are subject to a high conductance state during scratch in an isolated cord preparation in the turtle (Berg *et al.,* 2007). To determine whether the decreased AHP amplitude might result from a high conductance state during fictive scratch in the cat, conductance measurements were obtained throughout fictive scratch in both spinal intact and spinal transected conditions in 1 preparation. Figure 7A illustrates a SmAb motoneuron during fictive scratch as indicated by the MG and EDL ENG traces. The middle panel illustrates the rhythmic fluctuations in membrane potential (SDPs) with a portion of the trace expanded in the panel below. Hyperpolarizing current pulses (amplitude: 5 nA; duration 4.5 msec) were administered throughout fictive scratch (control pulses not shown). Figure 7B shows the membrane potential voltage deflection in control compared to the various phases of fictive scratch and recovery in a cell from a spinal intact and cell from a spinal transected preparation. The similarity of the voltage deflections between control and scratch conditions indicate that neither cell displayed large increases in conductance during fictive scratch.

Figure 8 shows the AHP of another SmAb motoneuron prior to (control) and during fictive scratch in a spinalized cat. The AHP is clearly visible during control (Figure 8B2), but not during fictive scratch (Figure 8B3). Figure 8C demonstrates that in this cell, conductance during fictive scratch was essentially the same as during control. Thus the AHP decreased in amplitude without an increase in cell conductance. Figure 9 shows the conductance measures of motoneurons during various phases of fictive scratch as a percentage of control. Changes in motoneuron conductance during fictive scratch ranged from a decrease of 35% to an increase of 34% compared to control. The most

noteworthy aspect however is that there is not a large and consistent increase in motoneuron conductance during fictive scratch. AHP amplitude reduction occurred for cells that demonstrated an increase, decrease, or no change in conductance during fictive scratch in the spinal intact and spinal transected preparations. Altogether, these data indicate that AHP amplitude reduction occurs in spinal-intact and spinal-transected decerebrate cat preparations during fictive scratch and is not due to a high conductance state.

Discussion

The most important finding in this study was that V_{th} hyperpolarization occurred during fictive scratch in decerebrate cats, both before and after acute C1 spinal transection. Threshold hyperpolarization was evident in both extensors and flexors with either high or low rheobases, did not exhibit phasic modulation, and was independent of rhythmic depolarizations produced by the scratch central pattern generator (CPG). In addition to V_{th} hyperpolarization, we demonstrated AHP amplitude reduction during fictive scratch before and after spinal transection and showed that this did not result from an increase in motoneuron conductance. V_{th} hyperpolarization and AHP amplitude reduction during fictive scratch will enhance motoneuron excitability and facilitate firing during motor output. Importantly, these effects occurred following spinal transection, indicating that an intraspinal mechanism is capable of modulating motoneuron excitability in the absence of supraspinal input.

Spinal-Intact – V_{th} hyperpolarization

Many studies have noted that V_{th} is not a fixed value. Kolmodin and Skoglund (1958) showed that the V_{th} depolarizes for successive action potentials occurring in a train, perhaps contributing to decreased firing rates induced by the inactivation of voltage-gated sodium channels. V_{th} hyperpolarization would tend to counter accommodation and ensure that motoneurons are recruited during motor output. Depolarization of V_{th} has also been demonstrated as a mechanism of training-induced spinal plasticity in the form of operant conditioning in monkeys (Carp & Wolpaw, 1994) and following decreased motoneuron activity as a result of hindlimb unweighting of adult

rats (Cormery *et al.*, 2005) and spinalization of adult cats (Hochman & McCrea, 1994) and rats (Beaumont *et al.*, 2004). Conversely, motoneuron adaptations following long-term sensitization training of the siphon withdrawal reflex in *Aplysia* included a hyperpolarization of their V_{th} (Cleary *et al.*, 1998) while 16 weeks of endurance treadmill training in adult rats induced V_{th} hyperpolarization of spinal motoneurons (Beaumont & Gardiner, 2003). All of these effects, however, were the result of prolonged changes in conditions.

Krawitz et al. (2001) provided the first description of an immediate and state-dependent V_{th} hyperpolarization. During fictive locomotion evoked by electrical brainstem stimulation in the decerebrate cat the V_{th} of motoneurons was hyperpolarized in the first evoked spike and recovered to control values within seconds of the end of locomotion. Gilmore and Fedirchuk (2004) found similar results during locomotor-like activity in an *in-vitro* neonatal rat brainstem spinal cord preparation. Both of these studies evoked motor activity by electrical brainstem stimulation. The presentation of V_{th} hyperpolarization during fictive scratch in adult decerebrate cats shows that V_{th} hyperpolarization does not require electrical brainstem stimulation. V_{th} hyperpolarization may be a more general mechanism used by the motor system to enhance motoneuron excitability in a variety of tasks.

Interestingly, V_{th} hyperpolarization also occurred for extensor motoneurons during the approach phase when extensors are normally quiescent, and during the hyperpolarized phase of the fictive scratch cycle. Threshold hyperpolarization was also evident in motoneurons that required intracellular current injection to initiate firing during fictive scratch, indicating that motoneuron firing is not required to initiate V_{th}

hyperpolarization. Despite the enhancement of motoneuron excitability, a portion of the motor pool remained un-recruited at that particular level of motor output.

Spinal-Transected – V_{th} hyperpolarization

Krawitz et al. (2001) suggested because V_{th} hyperpolarization occurred shortly after the onset of brainstem-stimulation-evoked fictive locomotion and did not recover immediately following its cessation, a neuromodulatory mechanism may be responsible. Further work by Gilmore and Fedirchuk (2004) demonstrated that during electrical brainstem-stimulation-evoked locomotor-like patterns in the *in-vitro* neonatal rat preparation, V_{th} hyperpolarization was eliminated upon cooling of the cervical spinal cord or through the addition of ketanserin to the bathing solution. These results suggest that the descending serotonergic pathway is at least one mechanism contributing to V_{th} hyperpolarization. Motoneurons in the spinal cord are innervated densely by monoaminergic input (serotonin: 5-HT; noradrenaline: NA) originating within the brainstem, known to have a potent effect on motoneuron excitability (for review see Rekling *et al.,* 2000). Given that the discharge frequency of the 5-HT-containing cells within the brainstem increases during repetitive types of motor output such as locomotion (Jacobs & Fornal, 1993), and that 5-HT exerts tonic facilitatory effects on spinal hindlimb motoneurons, it is likely that activation of these descending pathways during brainstem stimulation released monoamines within the spinal cord, enhancing motoneuron excitability. Indeed the ability of 5-HT and NA to induce V_{th} hyperpolarization in ventral horn neurons was demonstrated by Fedirchuk and Dai (2004) in the isolated *in vitro* neonatal rat spinal cord preparation.

Another well known effect of the monoamines on motoneuron excitability is their ability to facilitate PICs. PICs elicit plateau potentials in a variety of species and play important roles in amplifying synaptic input (Schwindt & Crill, 1995; Stuart & Sakmann, 1995), repetitive firing (Harvey *et al.*, 2006; Lee & Heckman, 2001), and action potential initiation during rhythmic firing (Kuo *et al.*, 2006; Lee & Heckman, 2001). Given that the monoamine systems are active during locomotion, it is not surprising that plateau potentials are evident during fictive locomotion in the decerebrate cat (Brownstone *et al.*, 1994). A series of studies by Hultborn and colleagues demonstrated bistability in α-motoneurons in the decerebrate cat in response to synaptic input or brief depolarizing pulses. This effect however was eliminated following spinal transection and recovered by 5-HTP or L-DOPA administration. They concluded that bistability was dependent upon active descending serotonergic and noradrenergic inputs (Crone *et al.*, 1988; Conway *et al.*, 1988; Hounsgaard *et al.*, 1988). Similar results following spinal transection in the decerebrate cat (Lee & Heckman, 2000) and *in-vitro* adult rat (Li *et al.*, 2007) have since been demonstrated. To our knowledge, similar studies have not been performed during fictive scratch, though voltage-dependent excitation in spinal motoneurons during fictive scratch has been demonstrated (Brownstone *et al.*, 1994; Perreault, 2002). However in these studies the animals were not spinalized.

Given that the monoaminergic systems are active during rhythmic motor outputs, are of predominantly supraspinal origin, drastically enhance motoneuron excitability, and induce V_{th} hyperpolarization in ventral horn neurons of rat, the question arises, "would V_{th} hyperpolarization be present during fictive scratch following spinal transection?" Our results demonstrate that V_{th} hyperpolarization occured during fictive scratch following

spinal transection rostral to the C1 spinal segment. Therefore, an intact spinal cord is not required for V_{th} hyperpolarization and mechanisms intrinsic to the spinal cord are capable of mediating this effect. We cannot conclude however that the descending monoaminergic pathways do not contribute to V_{th} hyperpolarization during fictive scratch. Although the spinal cord was transected at C1 thus eliminating the actions of active descending pathways, their axons are of course still present below the site of the lesion and may thus provide residual neuromodulatory input to the motoneurons. In the rat, acute spinalization does not eliminate PICs but reduces them to the point where they can no longer generate plateau potentials (Bennett *et al.*, 2001). Although spinal transection eliminates the majority of 5-HT and NA content in the spinal cord below the site of lesion, this can take on the order of days (Anden *et al.*, 1964). There is also evidence for a small population of intraspinal 5-HT containing neurons in the rat spinal cord following transection (Newton & Hamill, 1988). Thus, it is possible that the acutely spinalized spinal cord may still have monoaminergic input, albeit in reduced amounts. It is also possible that in the spinal-intact preparation, both spinal and supraspinal mechanisms contribute to V_{th} hyperpolarization. Without a comparision of the degree of V_{th} change between the spinal-intact and spinalized preparations we cannot say whether the lack of descending inputs can be noticed.

Afterhyperpolarization

In addition to V_{th} modulation, a reduction in the amplitude of the AHP was observed throughout the fictive scratch cycle, an effect that also occurred following spinal transection. Brownstone et al. (1992) demonstrated reduced AHP amplitude during

MLR-evoked fictive locomotion in the spinal intact decerebrate cat compared to the same cells in the non-locomotor state. Similar findings were obtained using the *in-vitro* neonatal rat spinal cord during locomotor-like activity, indicating that the regulation of AHP amplitude can be mediated in the absence of supraspinal input via intraspinal mechanisms (Schmidt, 1994).

A decrease in AHP amplitude could be the result of an increase in motoneuron input conductance and a passive "shunting" of the outward AHP current by simultaneously activated depolarizing inward currents. A high conductance state has been reported during scratch in turtle motoneurons (Alaburda *et al.*, 2005; Berg *et al.*, 2007). Alaburda et al. (2005) reported that the AHP of motoneurons in turtle slice experiments was dramatically reduced by even moderate increases in input conductance (e.g. 35%). They concluded that "the excitability and the intrinsic response properties of spinal neurons are periodically quenched by high synaptic conductance during functional network activity." These results however differ from those reported during motor output in neonatal rat (Schmidt, 1994), mouse (Endo & Kiehn, 2007), and cat (Shefchyk & Jordan, 1985). Schmidt (1994) demonstrated a decreased AHP amplitude in the presence of *decreased* input conductance during locomotor-like activity using an isolated neonatal rat spinal cord preparation. In the decerebrate cat, Shefchyck and Jordan (1985) reported that over half of the motoneurons examined showed no change in input resistance during fictive locomotion compared to control. Our results (Figure 9) are comparable to those of others during fictive scratch in the decerebrate cat (Perreault, 2002). Perreault (2002) reported an average decrease of more than 25% in input resistance in extensor motoneurons during fictive scratch, however of the 11 motoneurons in that study, not all

cells showed large decreases in input resistance during each phase of the scratch cycle. Thus, data from rat, mouse, and cat motoneurons do not support the existence of the high conductance state as described for turtle motoneurons (Berg *et al.*, 2007; Alaburda *et al.*, 2005). The fact that we have demonstrated decreased AHP amplitude occurring simultaneously with increases and/or decreases in input conductance during fictive scratch compared to the quiescent state, suggests that AHP amplitude reduction is not the result of a shunting effect, but is an active process that enhances motoneuron excitability. Activation of muscarinic receptors (M_2-type) has recently been shown to reduce AHP amplitude and increase motoneuron excitability during rhythmic motor output (Miles *et al.*, 2007). This would act to increase the output of the motoneuron and to facilitate repetitive firing. It remains to be determined whether cholinergic mechanisms underlie the AHP reductions that we observed during fictive scratch.

We suggest that the initiation of motor output places the spinal cord in a 'state' of enhanced excitability. This is not a new concept. It is well known that with the onset of fictive locomotion there is a reconfiguration of reflex pathways capable of switching from inhibitory to excitatory input. For example, extensor group I reflexes are predominantly inhibitory to extensors in the quiescent state (Jami, 1992) but when activated during locomotion, there is a reorganization of these reflexes, leading to excitation of the extensors. Thus, in addition to modulation of reflex pathways during motor output, there is a concomitant modulation of spinal motoneuron properties to enhance their excitability and facilitate firing, as previously discussed (see Introduction). V_{th} hyperpolarization would tend to counter the effects of accommodation that occurs during repetitive firing or with the initiation of firing on the depolarizing phase of SDPs.

Conclusion

We have demonstrated that V_{th} hyperpolarization and AHP amplitude reduction occur during fictive scratch in both spinal intact and spinal transected decerebrate cat preparations during fictive scratch. These state-dependent changes in the electrical properties of spinal motoneurons would act to ensure that motoneuron excitability is enhanced to facilitate firing brought about by the pre-motoneuronal circuitry producing alternating motor behaviours, as occurs in both fictive locomotion and fictive scratch and also to enhance motoneuron firing in response to sensory inputs during movement.

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Figure Legends

Figure 1. V_{th} is hyperpolarized during fictive scratch

A shows that fictive scratch was evident as rhythmic activity that alternated between extensor (GS) and flexor (TA) ENGs (top traces). The middle traces show spiking elicited by ramp-current injection (~ 7 nA) in control and without current injection during fictive scratch in a GS motoneuron (same time scale as upper traces). B and C are expanded views of the middle traces showing spiking and V_{th} values. B shows that the V_{th} for production of action potentials before fictive scratch was -38.4 mV and C shows that during fictive scratch, V_{th} for production of action potentials was -45.9 mV. This demonstrates a 7.5 mV hyperpolarization of V_{th}.

Figure 2. V_{th} is hyperpolarized during the approach phase

Panel A shows fictive scratch as rhythmic activity that alternated between extensor (GS) and flexor (TA) ENGs. Panels B and C show a trial for a GS motoneuron. Shaded areas labelled B and C denote the expanded time periods. Panel B shows the V_{th} for action potential initiation before fictive scratch was -36.5 mV. C shows that during the approach phase (note the ramp current injection occurs just prior to and throughout depolarization of the TA ENGs) the V_{th} for action potential initiation was -43.9 mV. This demonstrates a 7.4 mV hyperpolarization of V_{th}.

Figure 3. V_{th} is hyperpolarized throughout the fictive scratch cycle

Panel A shows extensor (GS) ENG activity during fictive scratch (top trace) and the intracellular membrane potential recording and current injection (middle traces) for a GS motoneuron. The shaded area labelled C denotes the expanded timescale in the lower traces (panel C). Panel B shows that the V_{th} for production of action potentials before fictive scratch elicited by a current-pulse injection (19.4 nA) was -39.4 mV. Panel C shows that a current pulse (27.8 nA) injected during the inhibitory phase of the fictive scratch cycle elicited an action potential with a V_{th} of -41.5 mV. This demonstrates a 2.1 mV hyperpolarization of V_{th}.

Figure 4. Fictive scratch before and after spinal transection

Panel 4A is an example of fictive scratch activity in a spinal intact cat. The top traces show the fictive scratch pattern as evident by a rhythmic alternation between flexor (Sart) and extensor (Sm) ENGs. The blue shaded area is an initial period of postural tonic flexion (Approach Phase) followed by motor output alternating between flexor (~ 200 ms) and extensor (~ 50 ms) activity (Rhythmic Phase – yellow traces). The bottom traces are an expanded view of ENG activity during fictive scratch and include: flexors (red), extensors (blue), and a bifunctional (purple). Panel 4B is an example of fictive scratch activity in the same cat as in panel A, but following an acute C1 spinal transection. Note the similar ENG activation patterns before and after spinal transection.

Figure 5. V_{th} is hyperpolarized during fictive scratch after spinal transection

Panel A shows fictive scratch as rhythmic activity alternating between extensor (SmAb) and flexor (Sart) ENGs (top traces) along with intracellular membrane potential recording and current injection (lower traces) in a GS motoneuron. The bar labelled C is an expanded area shown as Figure 5C. Panel B shows an action potential with a V_{th} of -48.6 mV elicited by a current pulse prior to fictive scratch activity. Panel C shows that during fictive scratch, spontaneous motoneuron firing (no current injection) was elicited with a V_{th} of -53.3 mV. This represents a V_{th} hyperpolariztion of 4.7 mV.

Figure 6. Afterhyperpolarization amplitude is reduced during fictive scratch

Panel 6A illustrates the same GS motoneuron recording as in Fig. 2. Ramp-current-evoked firing in control and during the approach phase of fictive scratch are compared (left and right panels, respectively). The bars below the voltage traces indicate the spikes from which the AHP average was obtained. Panels 6B and C are expanded views of the averaged AHPs for control and during fictive scratch. 6D is an overlay of panels 6B and C. The inset is the same traces aligned by their V_{th} values.

Figure 7. Conductance measurements during fictive scratch

The top traces in panel A are ENG recordings made during fictive scratch in a spinal intact cat. The middle traces are simultaneous intracellular recodings from a SmAb motoneuron in which hyperpolarizing current-pulses were injected throughout the bout of fictive scratch. The bottom traces are an expanded view. Hyperpolarizing current pulses are easily seen in the recording as negative deflections in the membrane potential. This

cell did not fire spontaneously during fictive scratch. Panel B shows an expanded view of averaged negative deflections in the membrane potential induced by the hyperpolarizing current pulses both before and after spinalization in the same cat. The averaged responses during each phase of the fictive scratch cycle (coloured) are superimposed on the control trace (black). Averaged recordings before and after spinalization are from different cells but are representative of the changes typically seen. In each of these cells V_{th} hyperpolarized and AHP amplitude was reduced.

Figure 8. AHP amplitude reduction without a change in motoneuron conductance

Top traces in panel A are ENG recordings made during fictive scratch. The middle traces are a simultaneous intracellular recording from a SmAb motoneuron during injection of hyperpolarizing current pulses. Spikes have been truncated. The bar labelled B1 represents region expanded in panel B1. Panel B is an expanded view of two spikes elicited by an intracellular current injection and their corresponding AHPs in the absence of fictive scratch (bar labelled B2 represents region expanded in B2). Prior to each depolarizing current-pulse, hyperpolarizing current-pulses were administered to assess changes in motoneuron conductance. Panel B1 is an expanded view of spiking during fictive scratch (bar labelled B3 represents region expanded in B3). During fictive scratch, hyperpolarizing current-pulses were injected to assess motoneuron conductance. Comparison of Panels B2 and B3, clearly demonstrates a reduction in AHP amplitude following each spike during fictive scratch (B3) when compared to control (B2). Panel C shows the averaged membrane potential deflection elicited by the hyperpolarizing

current-pulses injected during control (left trace) and the depolarized phase of the SDP (middle trace). The traces are overlaid to produce the right trace.

Figure 9. Histogram of conductance changes during fictive scratch

Bars represent the average conductance determined for each phase of the fictive scratch cycle in a sample of 10 motoneurons. Values are normalized to control (100% as indicated by the horizontal, dashed black line). Each phase of the fictive scratch cycle is represented by a different colour bar. The long, narrow, grey bar represents the split between motoneuron conductance assessment before and after spinal transection. All data are from one cat.

Figure 1

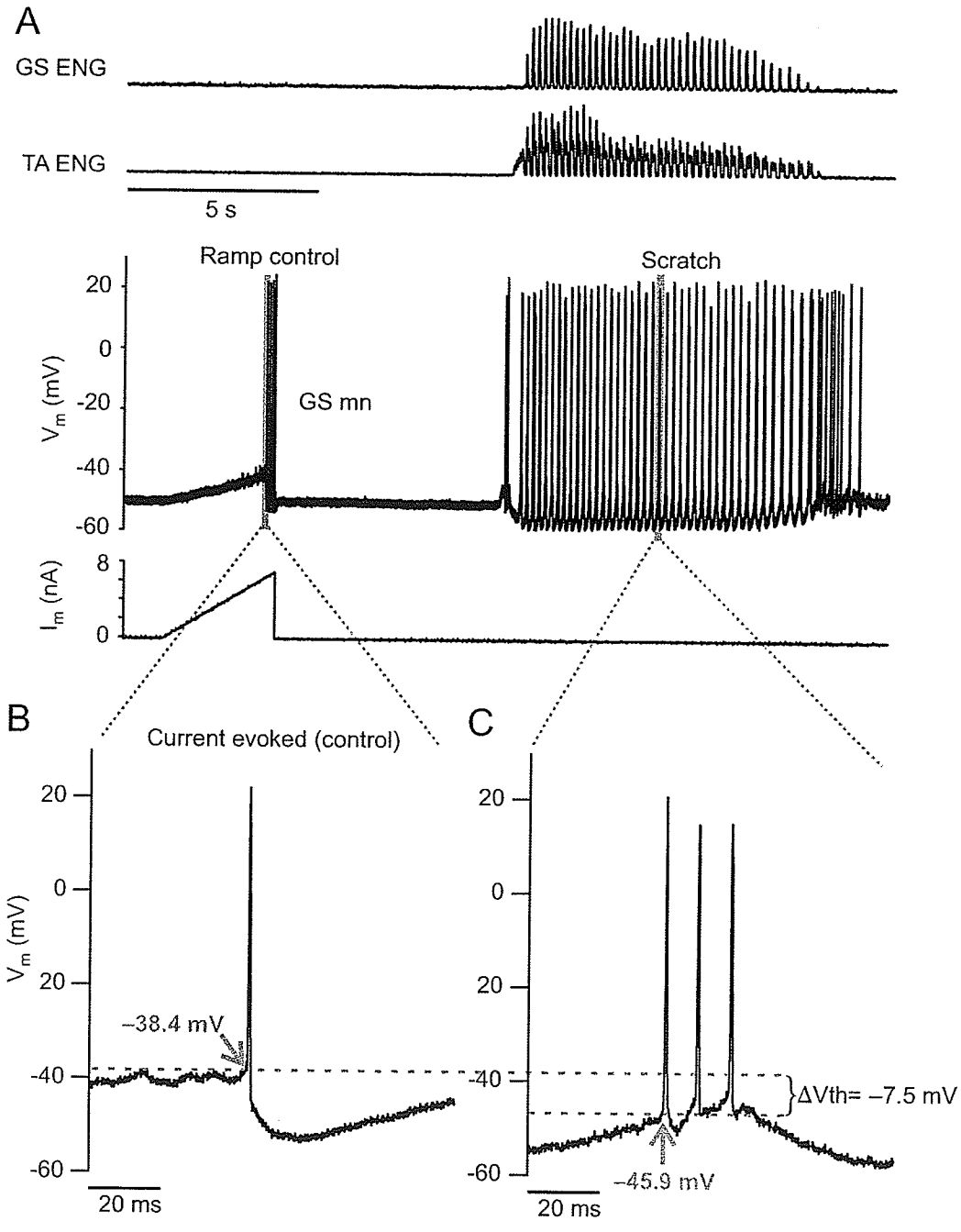

A
GS ENG
TA ENG
5 s
Ramp control
Scratch
GS mn
20
0
-20
-40
-60
V_m (mV)
8
0
I_m (nA)
B
Current evoked (control)
20
0
20
-40
-60
V_m (mV)
–38.4 mV
20 ms
C
20
0
20
-40
-60
ΔVth= –7.5 mV
–45.9 mV
20 ms

Figure 2

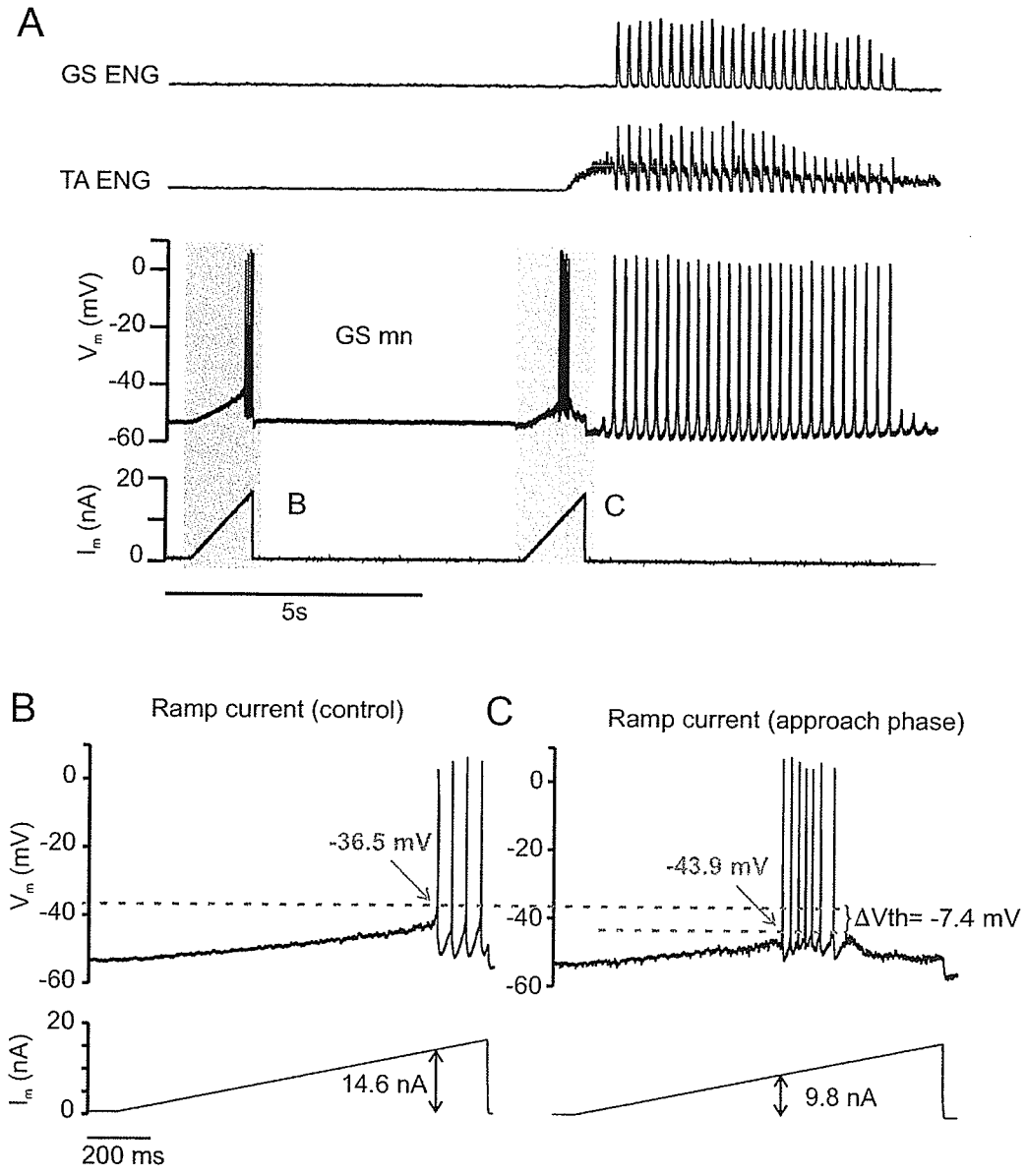

A
GS ENG
TA ENG
V_m (mV)
0
-20
-40
-60
GS mn
I_m (nA)
20
0
B
C
5s
B
Ramp current (control)
C
Ramp current (approach phase)
-36.5 mV
-43.9 mV
ΔVth= -7.4 mV
14.6 nA
9.8 nA
200 ms

Figure 3

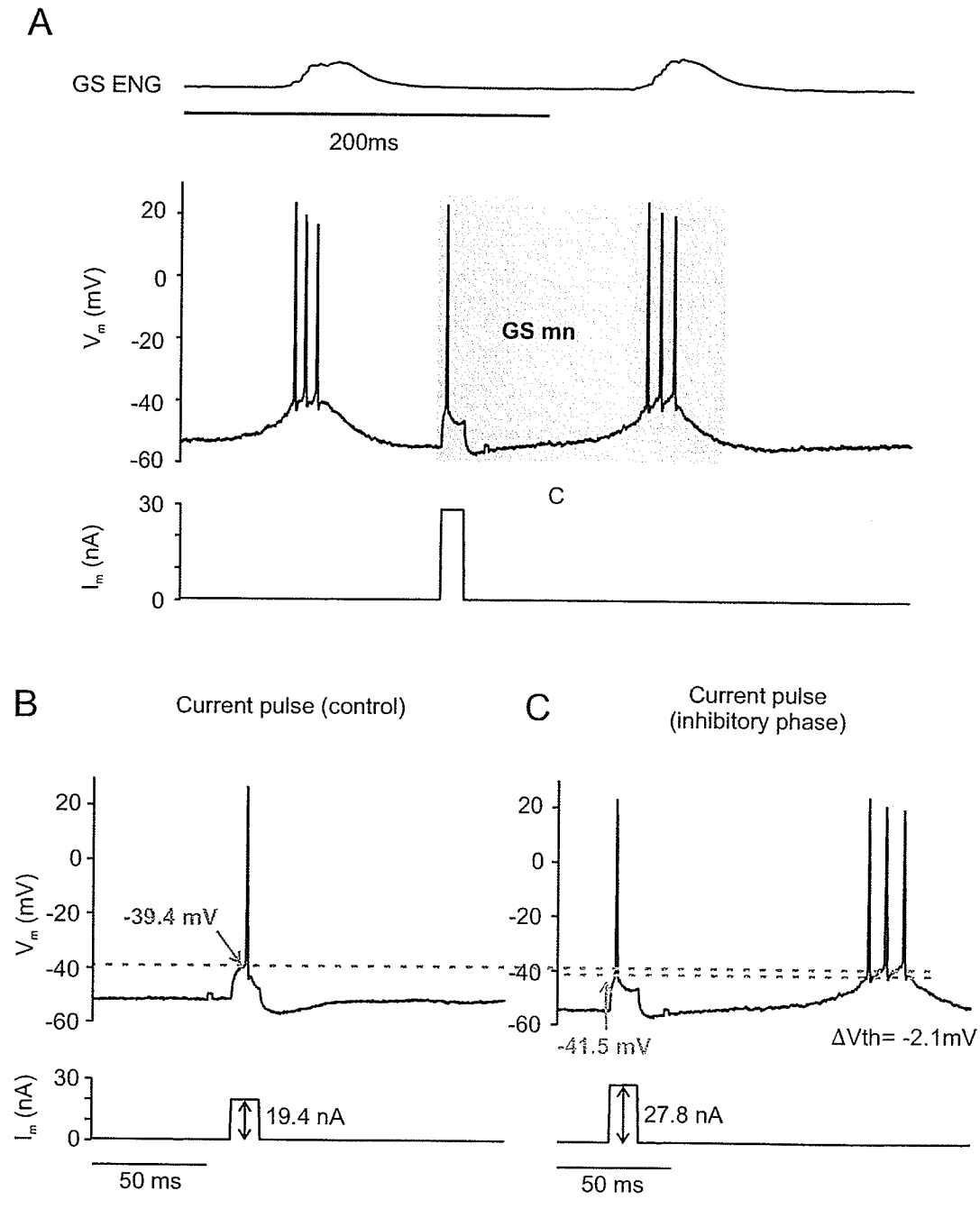

A
GS ENG
200ms
20
0
-20
-40
-60
V_m (mV)
GS mn
C
30
0
I_m (nA)
B
Current pulse (control)
C
Current pulse
(inhibitory phase)
-39.4 mV
-41.5 mV
ΔVth= -2.1mV
19.4 nA
27.8 nA
50 ms
50 ms

Figure 4

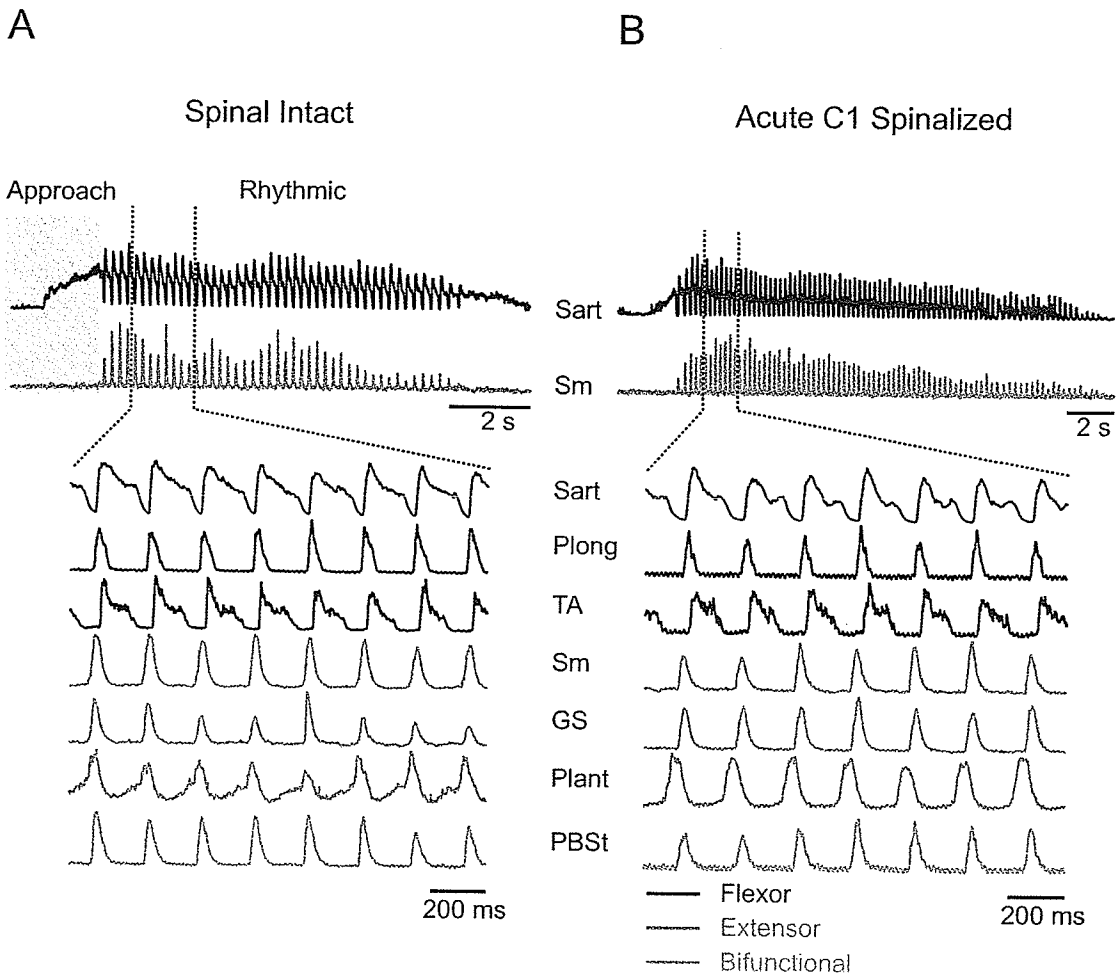

A
Spinal Intact
Approach
Rhythmic
2 s
200 ms
B
Acute C1 Spinalized
Sart
Sm
2 s
Sart
Plong
TA
Sm
GS
Plant
PBSt
Flexor
Extensor
Bifunctional
200 ms

Figure 5

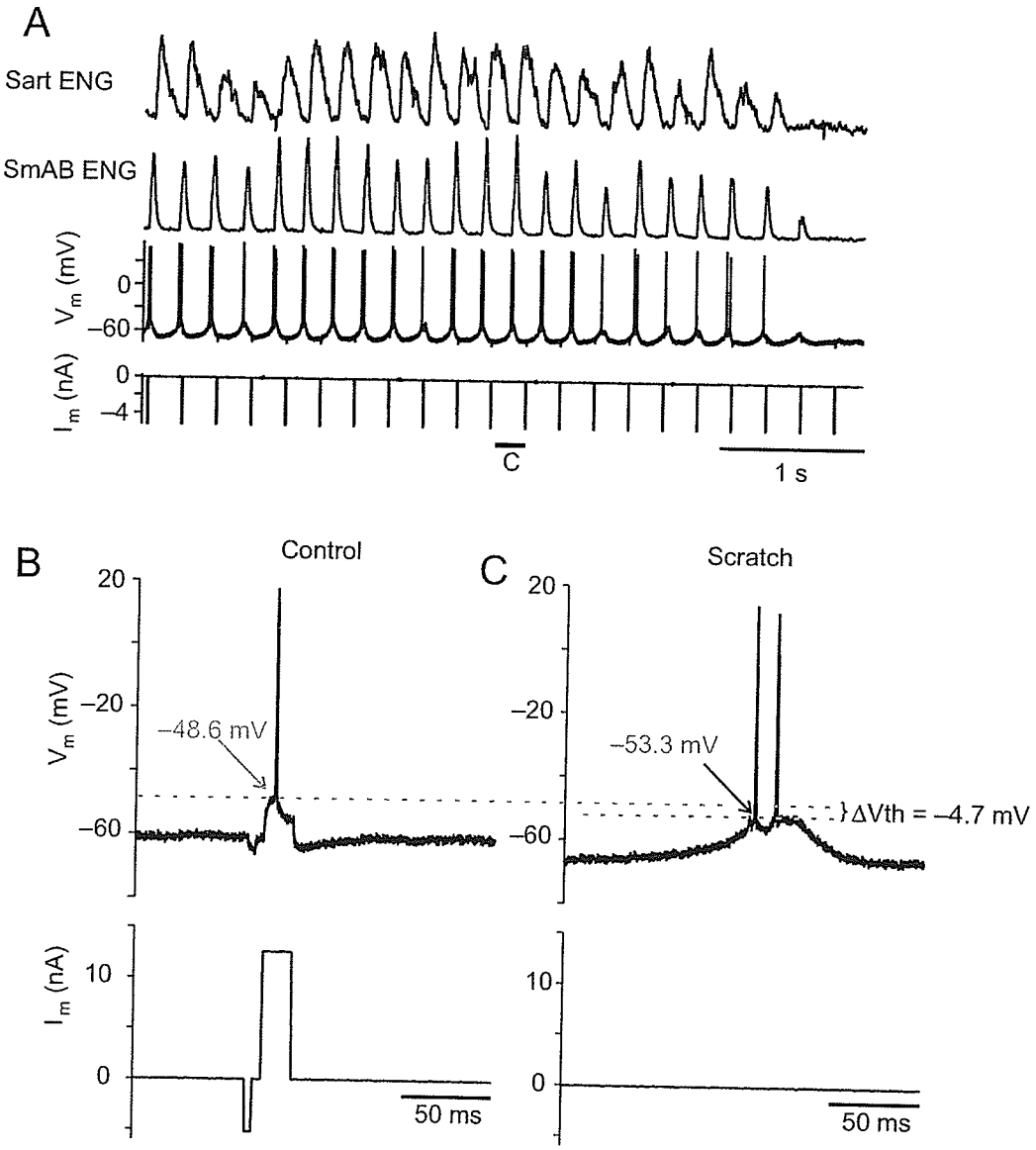

A
Sart ENG
SmAB ENG
V_m (mV)
0
–60
I_m (nA)
0
–4
C
1 s
B
Control
20
–20
–60
–48.6 mV
10
0
50 ms
C
Scratch
20
–20
–60
–53.3 mV
ΔVth = –4.7 mV
10
0
50 ms

Figure 6

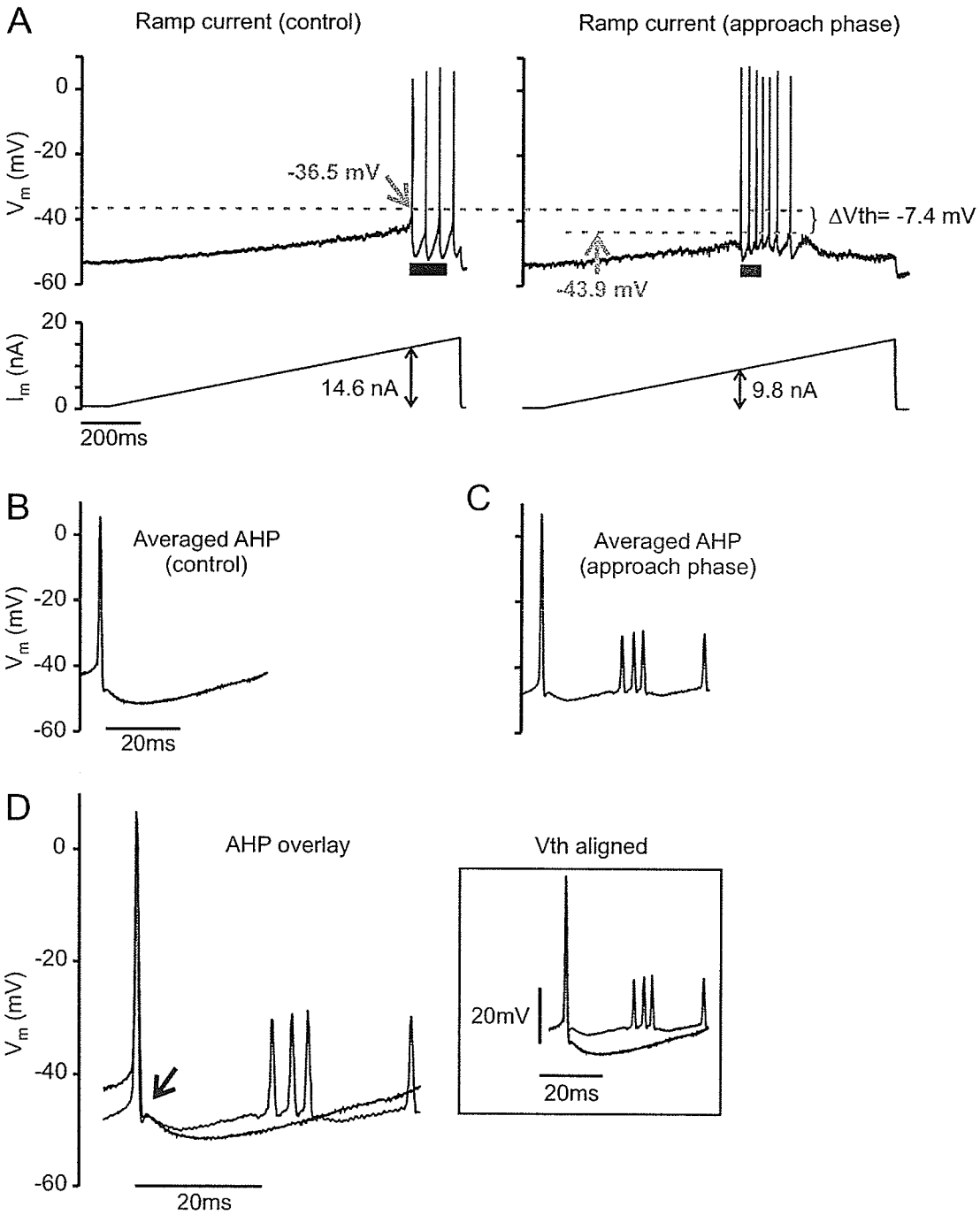

A
Ramp current (control)
Ramp current (approach phase)
0
-20
-40
-60
V_m (mV)
-36.5 mV
ΔVth= -7.4 mV
-43.9 mV
20
0
I_m (nA)
14.6 nA
9.8 nA
200ms
B
Averaged AHP
(control)
20ms
C
Averaged AHP
(approach phase)
D
AHP overlay
Vth aligned
20mV
20ms
20ms

Figure 7

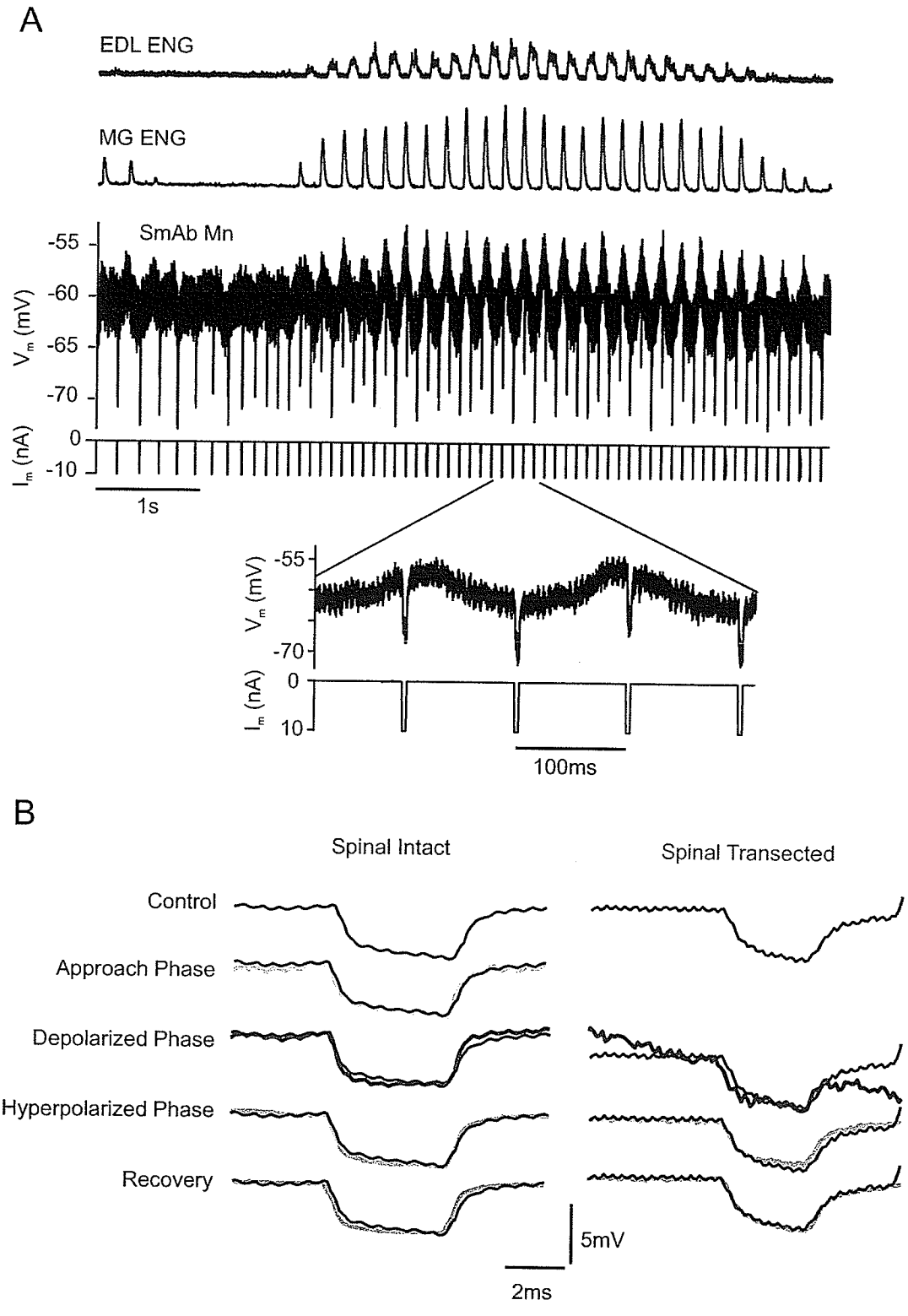

A
EDL ENG
MG ENG
SmAb Mn
-55
-60
-65
-70
V_m (mV)
0
-10
I_m (nA)
1s
-55
V_m (mV)
-70
0
I_m (nA)
10
100ms
B
Spinal Intact
Spinal Transected
Control
Approach Phase
Depolarized Phase
Hyperpolarized Phase
Recovery
5mV
2ms

Figure 8

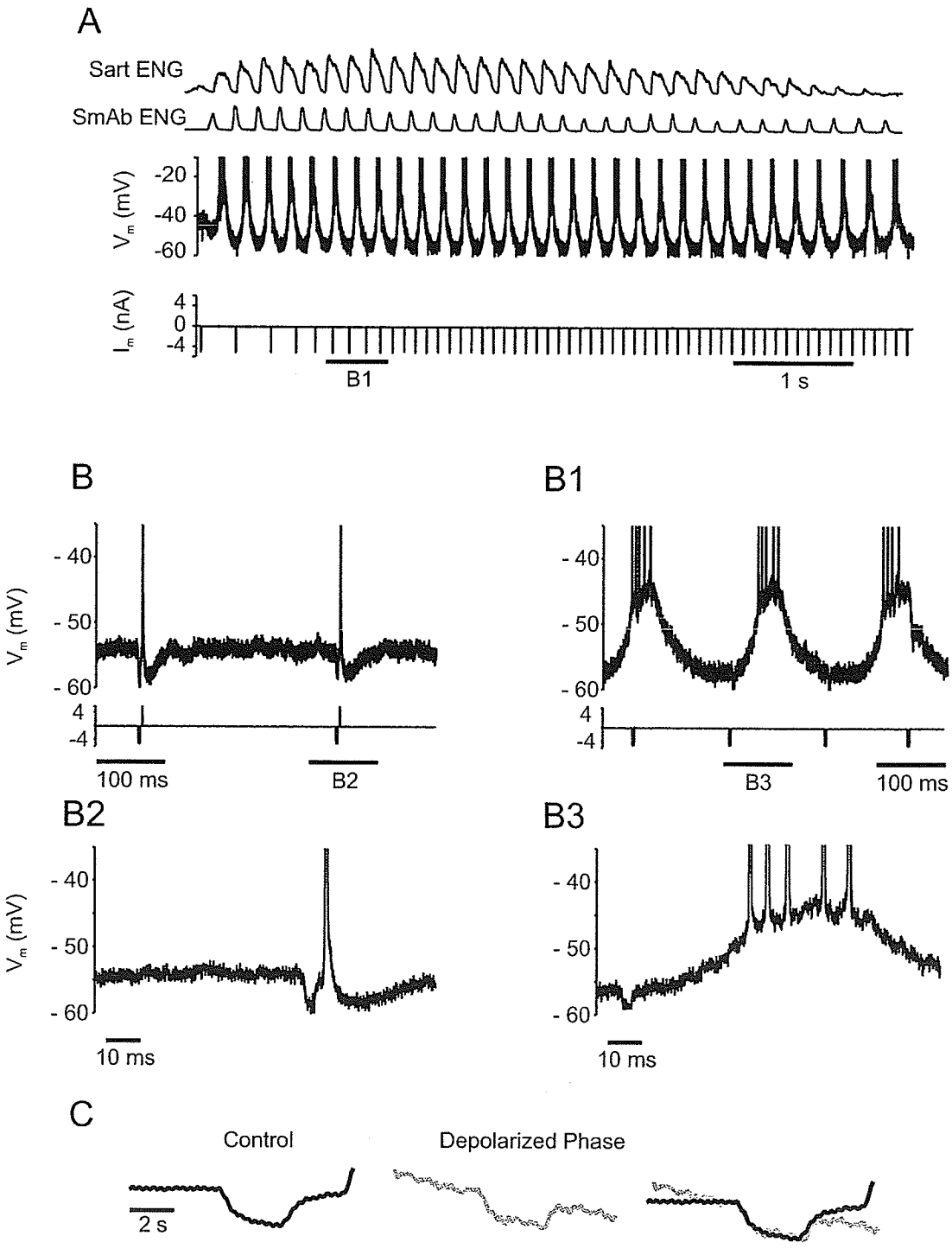
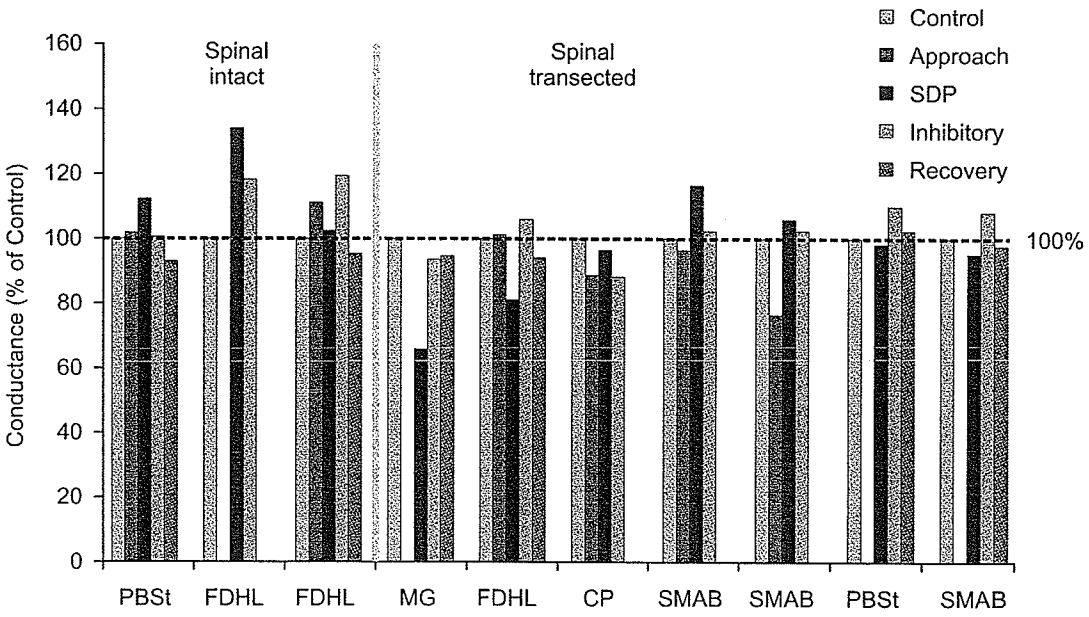

A
Sart ENG
SmAb ENG
V_m (mV)
-20
-40
-60
I_m (nA)
4
0
-4
B1
1 s
B
B1
- 40
- 50
- 60
4
-4
100 ms
B2
B3
100 ms
B2
B3
10 ms
10 ms
C
Control
Depolarized Phase
2 s

Figure 9


Control
Approach
SDP
Inhibitory
Recovery
Spinal intact
Spinal transected
Conductance (% of Control)
160
140
120
100
80
60
40
20
0
100%
PBSt
FDHL
FDHL
MG
FDHL
CP
SMAB
SMAB
PBSt
SMAB

IV. General Discussion

Project Goals and Findings

This thesis consists of two studies designed to: (1) understand the potential mechanisms by which V_{th} hyperpolarization occurs through pharmacological modulation of voltage-gated Na^+ channels (VGSCs), and (2) determine whether V_{th} hyperpolarization occurs during motor behaviours other than locomotion and whether it is dependent upon brainstem stimulation.

The most important finding in Paper 1 was that modulation of VGSCs induces V_{th} hyperpolarization in spinal motoneurons and that this effect is mediated through modulation of the I_{NaT} responsible for the upswing of the action potential and not by the I_{NaP}. The most important findings in Paper 2 were that motoneuron excitability is enhanced during fictive scratch via V_{th} hyperpolarization and decreased AHP amplitude, effects that occurred following acute C1 spinal transection.

Possible Mechanisms of V_{th} Hyperpolarization

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. Instead a brief review is presented in a logical order that may give insight by integrating the findings and implications of the studies that make up this thesis.

In the original description of V_{th} hyperpolarization, Krawitz et al., (2001) suggested that activation of the descending monoaminergic pathways may have been responsible for the observed V_{th} hyperpolarization. The ability of the monoamines to induce V_{th} hyperpolarization was subsequently reported using an *in vitro* neonatal rat

spinal cord preparation (Fedirchuk & Dai, 2004). Further work using a brainstem-attached *in vitro* neonatal rat spinal cord preparation demonstrated that locomotor-like activity initiated by electrical brainstem stimulation also induced V_{th} hyperpolarization, an effect blocked by a serotonergic antagonist (Gilmore & Fedirchuk, 2004). Given that the monoamines are capable of inducing V_{th} hyperpolarization, the question becomes, on which channel(s) are they acting to mediate this effect? As indicated in a modeling study, a likely candidate was the fast inactivating transient Na^+ channel mediating the action potential (Dai *et al.*, 2002). This provided the initiative for Paper 1 of this thesis. As discussed, we demonstrated that a negative shift in the voltage dependence of this Na^+ channel induced V_{th} hyperpolarization in the spinal motoneurons of an *in vitro* neonatal rat spinal cord. This suggests that the voltage sensors on the Na^+ channels become more sensitive to synaptic excitation generated during fictive locomotion, fictive scratch, and most likely other rhythmic motor outputs. We further suggest that monoamines may exert their effects on V_{th} through a similar action. We also conclude that facilitation of PICs, specifically the Na^+ portion of the PIC is unnecessary for V_{th} hyperpolarization. Of course we cannot exclude the possibility that the modulation of other channels in isolation or in combination with the NaT conductance may also mediate V_{th} hyperpolarization. More work is needed to study the effects of modulation on other conductances related to the generation of the action potential on V_{th}. As suggested by Dai et al. (2002) modulation of K^+ channels may also induce V_{th} hyperpolarization.

While the objective of Paper 2 in this thesis was not to elucidate possible mechanisms of V_{th} hyperpolarization, it did suggest some interesting possibilities. We showed that V_{th} hyperpolarization occurs during fictive scratch, in the absence of

electrical brainstem stimulation. This suggested that direct activation of the monoaminergic pathways may not have been necessary to induce V_{th} hyperpolarization. Perhaps mechanisms intrinsic to the spinal cord itself are capable of V_{th} modulation. The next logical step was to perform a spinal transection at C1, thus effectively isolating the spinal cord from all descending input, generate fictive scratch, and determine if V_{th} hyperpolarization occurred. It was. This further supported the idea that the spinal cord itself was capable of inducing V_{th} hyperpolarization during a motor output. We cannot conclude with certainty however that the monoamines are not inducing the demonstrated V_{th} hyperpolarization following spinal transection. There may still be residual monoamines in the spinal cord as a result of prior motor activity. In addition, the axons of these and other descending pathways are still present below the site of the lesion and may thus release monoamines within the spinal cord. However it is likely that there is at least a reduced input from the monoaminergic pathways. Others have shown that following spinal transection there is a loss of bistability and plateau potentials, characteristics of motoneuron excitability that are dependent on monoaminergic input from descending systems (Hounsgaard *et al.*, 1988; Crone *et al.*, 1988). The absence of plateau potentials following spinal transection lends indirect support to the finding in our first study that PIC enhancement is not necessary for V_{th} hyperpolarization. Another possibility however is that other transmitter systems, as yet unidentified, may act to induce V_{th} hyperpolarization independently or in combination with the monoamines. Acetycholine as an additional V_{th} modulator is discussed in more detail in the section entitled, "Additional questions and future directions."

State-dependent Changes in Motoneuron Properties

Regardless of the mechanisms responsible for V_{th} hyperpolarization, the fact remains that the motor system is capable of inducing changes in motoneuron excitability to facilitate motor output. Changes in motoneuron excitability during a motor output have collectively been referred to as "state-dependent," in that during a motor output the motor system is placed in a state of enhanced excitability. In addition to V_{th} hyperpolarization, previous reports have demonstrated the emergence of voltage-dependent excitatory conductances (Brownstone et al., 1994), a decreased rheobase current (Krawitz et al., 2001), and a reduction in the AHP amplitude (Brownstone *et al.*, 1992). A brief description of these state-dependent changes is given below.

Brownstone et al. (1994) showed that EPSPs in motoneurons originating from the locomotor CPG demonstrated non-linear changes in amplitude such that their amplitude increased with depolarization. In the same study, motoneurons were studied during fictive scratch to see whether the same effect was present. Depolarizing current pulses did not elicit plateau potentials during control, but did demonstrate voltage dependent excitation during the active phase of the fictive scratch cycle. Subsequent work demonstrated similar non-linear responses of EPSPs arising from muscle afferents in response to motoneuron membrane potential. This indicates that during motor output it is possible that there is an amplification of excitatory reflexes.

In the quiescent state, a reduction in AHP amplitude results in an increase in firing frequency or an increased slope of the frequency/current (f/I) relation. During fictive locomotion, the relationionship between AHP amplitude and firing frequency is lost in that the f/I normally becomes zero (Brownstone *et al.*, 1992). Brownstone et al.

(1992) suggested that AHP might not be involved in the regulation of repetitive firing. Further examination during fictive locomotion, however, has demonstrated that some cells maintain their f/I relation during different phases of the locomotor cycle (Fedirchuk et al., 1998). Dai et al. (2001) demonstrated in modeling studies that modulation of the initial segments Na^+ conductance through either a negative shift in the voltage dependency or an increase in maximum conductance could reduce the slope of the f/I curve and shift it to the left. This could partially explain the reduction of slope of the f/I curve during fictive locomotion.

V_{th} hyperpolarization during fictive locomotion indicates that less synaptic current is required to initiate an action potential, thus leading to enhanced excitability and possibly increased recruitment for force output. Dai et al. (2001) investigated the influence of V_{th} hyperpolarization of motoneurons on the output of entire motoneuron pools. They showed that a hyperpolarization of the V_{th} by 7 mV, a value similar to that seen during fictive locomotion and fictive scratch, resulted in increased recruitment of both S-type and F-type motoneurons. This would enhance the output of the motoneuron pool and likely decrease the synaptic input required via reflex or central pathways to recruit motoneurons to generate action potentials. It is also noteworthy that recent data from our lab have demonstrated that another means of modulating V_{th} is through activation of the PKC pathway, though the effect is mainly to depolarize V_{th}. Thus the implications are clear: V_{th} can be modulated in either a hyperpolarized or depolarized manner, depending on the receptor and pathway activated.

The findings of Paper 2 of this thesis provide further evidence for "state-dependent" changes in motoneuron excitability during fictive scratch, suggesting that V_{th}

hyperpolarization may be a common means by which the motor system enhances motoneuron excitability during rhythmic motor outputs. Given this finding, what are the implications of V_{th} hyperpolarization during motor output?

Possible Implications for State-dependent Changes in V_{th}

A hyperpolarization of the V_{th} for action potential initiation in combination with various other changes in motoneuron properties during motor output has important consequences for motoneuron recruitment and firing. Less depolarization required to initiate firing implies a decrease in the amount of required synaptic current, which in turn enhances the ability of EPSPs generated via central or reflex pathways to initiate action potentials. This could substantially decrease the number of interneurons required to excite the motoneurons and lead to easier motoneuron recruitment.

During sustained supra-threshold input, motoneurons exhibit a time-dependent decrease in action potential firing rate. This process is called 'spike frequency adaptation' (SFA) and has been suggested to play a role in fatigue (see Kernell, 2006). SFA is characterized by 3 phases, initial, early, and late. Several mechanisms have been proposed to contribute to SFA including a summation of the AHP, which is mediated via a Ca^{2+} activated K^+ conductance, and inactivation of the VGSCs contributing to both the I_{NaP} and I_{NaT}. Recent work using an *in vitro* mouse slice preparation, however, suggests that early adaptation is most likely due to slow inactivation of the Na^+ channels mediating the I_{NaT} (Miles *et al.*, 2005). Given that the initiation of motor output induces V_{th} hyperpolarization and that we propose that this effect is mediated by the same conductance suggested by Miles et al. (2005) to be responsible for early SFA (NaT), it is possible that during motor output motoneurons will not undergo SFA to the same extent

as they would in its absence. Indeed, Krawitz (2005) demonstrated that late adaptation is abolished during fictive locomotion. It is possible that V_{th} hyperpolarization, perhaps mediated by effects on VGSCs during motor output, negates the decrease in firing frequency associated with SFA, thus increasing motoneuron excitability and enhancing the ability of spinal motoneurons to fire at higher rates for a longer period of time, possibly reducing the effects of fatigue.

Experimental Limitations

In Paper 1 of this thesis we used the *in vitro* neonatal rat preparation to study V_{th} hyperpolarization. The spinal cord and motor systems of neonatal rats are not fully developed (for more detail see General Introduction) and thus may be perceived as a limitation to the study. However the purpose of the study was to reproducibly induce changes in V_{th} as a result of pharmacological modulation rather than to ascribe absolute values for V_{th} to mature animals using a neonatal preparation.

A second potential limitation is the fact that we are measuring V_{th} in voltage clamp. Thus, we are in fact measuring the current threshold to elicit a spike and determining the V_{th} as the voltage step utilized to elicit that current. This is perhaps not so much an experimental limitation as it is merely terminology, yet it may cause some confusion. It is noted however that this terminology is used to describe this phenomenon. This brings about another potential issue. In applying depolarizing voltage steps to initiate an inward current, the protocol uses 2 mV steps. This would allow for changes in V_{th} less than 2 mV to go undetected.

In our second study an obvious technical challenge and experimental limitation involves our inability to effectively quantify the AHP amplitude during fictive scratch,

the details of which were discussed in that manuscript. Our final approach was to semi-quantitatively determine if AHP amplitude was reduced during fictive scratch. While it would be beneficial to report AHP amplitude values, our approach relied on the fact that a reduction in its amplitude during fictive scratch was the most important finding, not the absolute amount of change.

Additional Questions and Future Directions

V_{th} hyperpolarization and its mechanisms are far from being completely understood. Many questions still remain. For example, which receptors are mediating this effect? What are the intracellular signaling pathways responsible? Does V_{th} hyperpolarization occur only during rhythmic movements or is it present during other types of motor outputs? Are interneuronal populations also subject to V_{th} hyperpolarization?

A question that is of great interest to me is whether there is evidence of plateau potentials or PICs during fictive scratch in a spinal transected decerebrate cat. In Paper 2 of this thesis we demonstrated that V_{th} hyperpolarization during fictive scratch does not require electrical brainstem stimulation or an intact spinal cord. Given that previous work has suggested that the monoamines are capable of inducing V_{th} hyperpolarization (Fedirchuk & Dai, 2004), that their origin is of supraspinal location (Dahlstrom & Fuxe, 1965; Gilman, 1992) and that they facilitate plateau potentials (Hounsgaard & Kiehn, 1985) it would be beneficial to determine whether there is evidence for PICs during fictive scratch before and after spinal transection. Based on previous work in the cat, I would hypothesize that PICs are not present during fictive scratch following spinal transection. If in fact this were the case, this would have two implications: (1) it would

further strengthen our finding in Paper 1 of the thesis that PICs are not necessary to induce V_{th} hyperpolarization; and (2) it would confirm that in addition to the descending monoaminergic pathways, mechanisms intrinsic to the spinal cord are capable of inducing V_{th} hyperpolarization. To test this hypothesis, one could inject depolarizing current ramps into spinal motoneurons during fictive scratch after spinal transection. Ideally this would be performed with QX314 in the recording electrode to block spiking, thus providing the ability to determine if voltage-dependent excitation occurs during the SDPs. This would be evident in an increased size of the SDPs at more depolarized membrane potentials.

Another important question is, "Are there additional transmitters that mediate V_{th} hyperpolarization, such as acetylcholine (ACh)?" This seems to be quite an interesting possibility. Reduced AHP amplitude during motor output was first demonstrated during fictive locomotion in the decerebrate cat (Brownstone *et al.*, 1992). Further work using the isolated *in vitro* neonatal rat spinal cord demonstrated AHP reduction during locomotor-like activity, indicating intraspinal mechanisms were mediating this effect (Schmidt, 1994). Our results during fictive scratch support this finding. Recently Miles et al., (2007) demonstrated that cholinergic activation of M_2-type muscarinic receptors decreased the AHP amplitude in the spinal motoneurons of mice. Moreover, they showed that the C boutons containing ACh originated in a population of interneurons located lateral to the central canal. These findings may explain the reduced AHP amplitude seen during fictive scratch following spinal transection. If ACh also induces V_{th} hyperpolarization, this would provide an explanation for V_{th} hyperpolarization following

spinal transection. Therefore, the induction of V_{th} hyperpolarization and reduction of AHP amplitude following spinal transection may be mediated by the cholinergic system.

Finally, an interesting question is whether the state-dependent changes are limited to motoneurons. Various populations of spinal interneurons project to motoneurons thus contributing to the regulation of motoneuron excitability. An important question becomes, "Are spinal interneurons subject to V_{th} hyperpolarization and AHP reduction during motor output?" To my knowledge, this has not been investigated. If this were the case, this would lend further support to the concept of a state-dependent change in spinal excitability.

Final Thoughts

Motor activity depends on a combination of factors including reflexes and intrinsic spinal circuitry. Spinal reflex systems alter motoneuron output via direct (monosynaptic) and indirect (polysynaptic via interneurons) synaptic connections. These reflex pathways are modified under different conditions as seen for example during a "reflex reversal" or "resetting." While a detailed discussion of the state-dependent modulation of segmental reflexes is beyond the scope of this thesis, it is important to acknowledge that there are changes in the motor system extrinsic to the motoneuron itself contributing to their excitability. In this thesis we investigated V_{th} hyperpolarization, which is one part of a much bigger picture involving state-dependent changes in spinal cord function during motor output. V_{th} hyperpolarization is a relatively new discovery with many details yet to be described. The strength of this thesis rests in two main findings: (1) an enhanced Na^+ channel activation can induce V_{th} hyperpolarization; and

(2) intraspinal mechanisms are capable of inducing V_{th} hyperpolarization independent of supraspinal input.

To generate movements, motoneurons must integrate the synaptic inputs they receive to produce an output, via the nerve, to the muscle for contraction. Following spinal cord injury the descending commands required to initiate these movements are lost or impaired. Much attention has recently been focused on the regeneration of descending axons in an attempt to 'repair' these damaged connections. If or when these efforts are realized, this does not guarantee recovery of function. An understanding of the mechanisms controlling motoneuron excitability will be essential for strategies to facilitate functional recovery.

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