The excitability of lumbar motoneurons in the neonatal rat is increased by a hyperpolarization of the voltage threshold for activation by descending serotonergic fibers.

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In Partial Fulfillment of the Requirements for the Degree of

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By

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The Excitability of Lumbar Motoneurons in the Neonatal Rat is Increased by a Hyperpolarization of the Voltage Threshold for Activation by Descending Serotonergic Fibers

BY

Jonathan Gilmore

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

Contents

I went to the woods because I wished to live deliberately, to front only the essential facts of life, and to see if I could not learn what it had to teach, and not when I came to die, discover that I had not lived.... I wanted to live deep and suck out all the marrow of life, to live so sturdily and Spartan-like as to put to rout all that was not life, to cut a broad swath and shave close, to drive life into a corner, and reduce it to its lowest terms, and if it proved mean why then to get the whole and meanness of it...or if it were sublime, to know it by experience.

Give me a hammer, and let me feel the furrowing. Drive a nail home and clinch it so faithfully that you can wake up in the night and think of your work with satisfaction, - a work at which you would not be ashamed to invoke the Muse. Every nail driven should be as another rivet in the machine of the universe, you carrying on the work.

Henry David Thoreau - Walden

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Abstract

The recent discovery of a state-dependant change in Voltage threshold (Vth) has opened a new door into the understanding of neuronal excitability. Recent work by Krawitz et al. (2001) has shown that there is an increase in motoneuron excitability produced by the hyperpolarization of the threshold potential at which an action potential is elicited during fictive locomotion the decerebrate cat. This phenomenon represents an instantaneous modulation of Vth dependant on fictive locomotion. The present study builds upon the work of Krawitz by reporting that electrical stimulation of the neonatal rat brainstem or dorsal roots can produce Vth hyperpolarization independent of measurable ventral root output. In addition hyperpolarization of Vth was seen in 10/11 (range –2 to –18mV) neurons recorded during locomotor-like ventral root activity. This appears to be similar to the locomotor-dependant Vth hyperpolarization described by Krawitz. Vth hyperpolarization was seen during electrical brainstem stimulation that evoked ventral root activity including; alternating, rhythmic, and tonic or even during no ventral root activity. Thirty-six of 71 neurons were antidromically identified as lumbar motoneurons and 33/36 showed a hyperpolarization of Vth (-2 to –14mV) during electrical brainstem stimulation. Thirty-one of 35 of the identified motoneurons also showed hyperpolarization of Vth (-2 to –20 mV) during brainstem stimulation. Cooling of the cervical cord reversibly blocked Vth hyperpolarization and ventral root-output, indicating that the Vth hyperpolarization is mediated via descending fibers. Experiments with 5-HT_{2A} receptor antagonists indicate that they are involved in Vth modulation. Vth hyperpolarization is hypothesized to be a mechanism that is used as a way to increase motoneuron excitability pursuant to a motor output.

List of Abbreviations

Vth	Voltage Threshold
CPG	central pattern generator
MLR	mesencephalic locomotor region
CNS	Central nervous system
ENG	electroneurogram
EMG	electromyogram
VR	ventral root
IaIn	Ia inhibitory interneurons
LDP	Locomotor Drive Potential
BES	Brainstem electrical stimulation
VMM	Ventromedial Medulla
MED	MedioVentral Medulla
5-HT	Serotonin
Ach	Acetylcholine
NE	norepinephrine
NMDA	N-methyl-D-aspartate
EAA	excitatory amino acid
C	cervical
T	thoracic
L	lumbar
S	sacral
lL2	left lumbar root two
rL2	right lumbar root two
lL5	left lumbar root five
rL5	right lumbar root five

Introduction

In 2001 Krawitz et al. published a paper that introduced a new phenomenon in neuroscience that described a state-dependant hyperpolarization of voltage threshold (Vth) in the fictive locomoting cat. A hyperpolarized Vth is present when an action potential is activated at a membrane voltage more negative compared to control. The phenomenon of Vth hyperpolarization is a newly described way of increasing excitability of neurons.

In order to examine the phenomenon of motoneuron Vth hyperpolarization during locomotion and some of the neurotransmitter mechanisms responsible for the process, the *in vitro* neonatal rat brainstem/spinal cord preparation was employed. In the present experiments electrical stimulation of the brainstem was used to produce a locomotor-like output without the addition of neurotransmitte.

This introduction briefly reviews the history, models and localization of the central pattern generator (CPG) for locomotion in mammals. Many different models have been developed in order to study locomotion and its mechanics, including: cat, rat, leech, newt, salamander, turtle, lamprey, and recently mouse. The introduction reviews the mammalian models for locomotion as well as pharmacological and electrical strategies for evoking locomotion. The introduction then reviews of some of the evidence that cellular properties are altered during locomotion; and that the membrane potential for action potential initiation in a single neuron is not a fixed value.

History of the Concept of the Central Pattern Generator

In all vertebrate models locomotion involves the coordination of rhythmic alternating activity of antagonist muscle groups, controlled by neuronal circuits that exist

within the spinal cord. Since the turn of the century, researchers have been aware that locomotion could be elicited from animals lacking cortical, brainstem or cerebellar structures. Visual observations of 'reflexive stepping' could be elicited from chronic spinal cats, demonstrating that locomotor movements could be organized without descending input from the brain or brainstem (Sherrington 1906). Sherrington hypothesized the locomotor movements he observed were due to a series of reflexes. However, Brown (1911) observed spontaneous alternation of muscle contractions in antagonist hind limb muscles of deafferented cats, suggesting that the activity was spontaneous and not triggered by a reflex. Thus, the idea that locomotion was centrally programmed within the spinal cord and not a series of reflexes originated. The intrinsic neuronal circuitry needed for locomotion has been termed the CPG (Grillner 1981) for locomotion.

Central Pattern Generator Models

It is not known at this time whether there is a group of spinal neurons dedicated to locomotion or a population that is able to contribute to many different behaviours (e.g. locomotion and scratch), although it is likely that the CPGs for scratch and locomotion share some neuronal populations (Grillner 1981). Attempts to conceptualize the CPG have resulted in several models of the CPG being proposed.

Brown (1914) hypothesized that a neural unit organized as "the half center" could produce alternation between flexor and extensor motor units. Conceptually, one half of "the neural unit" controls extensors while the other half is dedicated to flexors. Each "half center" is connected to the other by mutually inhibitory recurrent motor axon collaterals (Brown 1914). This model (Fig1A) assumes that the two units receive equal

excitation, but that higher activity in one pool inhibits the other and thereby leads to decreased inhibition in the already dominant pool. The dominant pool eventually becomes refractory and the alternate pool then assumes dominance, this alternation between pools repeatedly cycles, producing locomotor activity.

Jankowska et al. (1967a) used intracellular recordings of motoneurons and extracelluar recordings in cat spinal cord to verify the mutually inhibitory reflex pathways described by Brown (1914). However researchers found that that the reciprocal connections between flexors and extensors were composed of inhibitory interneurons and not recurrent axon collaterals (Jankowska et al. 1967a) (Fig. 1B).

Kling & Szekely (1968) proposed the "ring" model of the CPG. The ring composed three neurons arranged in a ring with each neuron inhibitory to one another. If cell A was firing it would inhibit cell C, cell C then became silent and cell C stopped inhibiting cell B which was inhibitory to cell A. When cell B started firing it would inhibit cell A. Cell A then became silent and cell C becomes active. The cycle length was determined by the time from when the inhibition stops until the cell became active. Each of the cells can be connected to motoneurons and cause rhythmic activity (Figure 2A).

In the swing generator model proposed by Pearson (1976), he suggested a network that generated flexor bursts (Grillner 1981). Pearson concluded that the activated network components producing flexion were active for a set duration and that extension followed flexor activity. Pearson's (1976) model (fig. 1D) was composed of interneurons that periodically produced bursts of activity in motoneurons during swing (flexor) and inhibited motoneurons active during stance (extensor). Activation of the

swing generator was via central or reflex inputs and remained active for a set period. Disinhibition could activate the extensor motoneurons, and resulted in an alternation between flexion and extension (Grillner 1981).

Miller & Scott (1977) proposed a model of the CPG using Ia inhibitory interneurons (IaIn), Renshaw cells, and antagonist motoneurons. The model generates alternating inhibition of flexor and extensor motoneurons via phasic IaIn inhibition of motoneurons (Grillner 1981). The pattern of IaIn activity is determined by the inhibitory action of the Renshaw cells. The rhythmic alternations between flexors and extensors is produced by the delay associated with the Renshaw cell recurrent pathway and its influence on IaIns (Fig. 1C)

The Central Pattern Generator for Locomotion is Distributed Within the Spinal Cord

The existence of a spinal cord locomotor CPG has been accepted for some time, yet the exact localization of cell populations within the spinal cord constituting the CPG is still debated. Initial experiments lead some research groups to believe that the CPG for locomotion may be restricted to certain segments of the spinal cord. Noga et al. (1995) determined that in the decerebrate cat, stimulation of the mesencephalic locomotor region (MLR) evokes fictive locomotion by activating interneurons in lamina VI - X, predominantly between segments L4 and L6. Cazalets et al. (1995) administered N-methyl-D-aspartate (NMDA) and Serotonin (5-HT) to separate segments of the neonatal rat spinal cord and monitored ventral root recordings. They found that L1/L2 produced locomotion at a concentration of 5-HT that did not produce locomotion when applied to other segments and concluded that the CPG for the hind limbs of the neonatal rat was

restricted to the L1/L2 segments. The idea of a restricted CPG, although convenient, has generated controversy in the literature based on further investigation of CPG localization.

Cowley & Schmidt (1997) showed administration of 5-HT and NMDA to any segment of the cervical and thoracic cord of the neonatal rat could produce rhythmic electroneurogram (ENG) discharge. Contrasting Cazalets (1995), Cowley & Schmidt (1997) concluded that the 5-HT sensitive network for hind limb locomotor-like activity was distributed in the supra-lumbar segments of the spinal cord and mediates descending rhythmic drive to the lumbar segments. Cowley & Schmidt (1997) concluded that there are NMDA and Acetylcholine (Ach) sensitive regions distributed throughout the spinal cord, and addition of these agents to any segment of the cord could produce ventral root output. Further they stated that midsagittal lesion of the lumbar segments demonstrated that this network is redundant, propriospinal and contains a system of reciprocal excitatory and inhibitory cross projections.

In an effort to localize the regions of primary importance to the CPG through rhythm and pattern generation, Kjaerulff & Kiehn (1996) lesioned discrete regions of the neonatal rat spinal cord and induced locomotion using 5-HT and NMDA. They found that the rhythm-generating network was distributed throughout the entire lumbar cord and extended into caudal and thoracic regions. They also found that the pathways mediating left/right alternation exist primarily in the ventral commissure. They concluded that the neuronal populations for the CPG are ventral to the central canal and are located more medially in the spinal cord.

Kjaerulff et al. (1994) labeled cells with sulphorhodamine during 5-HT and NMDA induced locomotor activity in the neonatal rat. They found high labeling in the

region around the central canal and in the intermediate grey area (laminae VI-VII). Kjaerulff et al. (1994) felt that these neurons were responsible for the CPG. However, Cina & Hochman (2000) reported there was false positive labeling when inducing locomotor activity using 5-HT and NMDA. They found that if the locomotor rhythm was evoked using only 5-HT, that sulforhodamine-labeled neurons were located predominantly in lamina VII.

Tract-tracing experiments in the neonatal rat showed that ascending axons passing through the ventrolateral funiculus (VLF) had somata located in lamina VII from T13-L4 (Antonino-Green et al. 2002). The peak number of cells was located in L2-L3. In the cat laminae VI-X (Noga 1994) and laminae VI-VII (Kiehn 1994) may contain populations active during fictive locomotion. Intracellular recordings of laminae VII neurons shows they are rhythmically active during chemically-evoked locomotor-like activity in the neonatal rat spinal cord (MacLean et al. 1995).

Ballion et al. (2001) investigated the spinal localization of the neonatal rat forelimb CPG. They found that a locomotor rhythm could be elicited from C7 - T1 with high K^+ artificial Cerebral Spinal Fluid (aCSF). A stronger pharmacological activation of the cervical thoracic cord with (5-HT and NMDA) could evoke locomotor-like bursting through C3-L2. These results lead Ballion et al. (2001) to suggest that an intrinsic rhythmic capacity exists at cervical thoracic levels.

The accumulated evidence suggests that the elements of the CPG for locomotion are distributed throughout the spinal cord with segments innervating limbs showing greater ability to generate rhythmic output and that more ventral and medial portions of the spinal cord contain the neuronal circuitry for generating left/right-alternating activity.

Mammalian Models for Spinal Cord Study

The *In vivo* Cat Preparation

The *in vivo* cat provides an adult preparation with fully-developed motor and sensory systems. Nerves innervating hind limb muscles can be dissected to allow a very precise recording of motor activity. This preparation also has well-defined inputs to interneurons and motoneurons allowing examination of afferent, interneuron, and motoneuron activity during locomotion and scratch. The preparation however, does have limitations. As an *In vivo* preparation, modification of the spinal cord extracellular fluid is difficult. The blood/brain barrier can interfere with pharmacological agents and their site of action. While experiments using iontophoresis can circumvent the latter problem these are technically challenging experiments.

The *in vitro* Neonatal Rat Preparation

The development of the *in vitro* neonatal rat brainstem/spinal cord preparation by Otsuka & Konishi (1974) has become a powerful preparation to study mammalian spinal cord physiology. The *in vitro* neonatal rat cord can survive for hours in a dish of aCSF oxygenated with 95% O_2. Due to the small size of the spinal cord, diffusion delivers oxygen to the cells of the spinal cord. The neonatal *in vitro* preparation has many advantages for the study of Vth hyperpolarization. The composition of the extracellular medium can be controlled and there is improved stability for intracellular recordings with the absence of respiratory movements or blood pressure. Similar to the decerebrate cat preparation, the animal is decerebrated (removes the need for ongoing anesthesia), and deafferented (releasing the systems from descending control and afferent input). The model allows control of the extracellular medium. The preparation also allows the

convenient addition and wash-out of pharmacological agents. The addition of pharmacological agents to the bath is easy and precise, agents can access their site of action quickly due to incomplete development of the blood/brain barrier. One limitation of bath application of pharmacological agents however is that because of the diffusion barrier the concentration of the agent within the spinal cord tissue is unknown.

The *in vitro* preparation relies on diffusion of gases to the tissue from the aCSF to meet metabolic demands for oxygen. Inadequate diffusion can result in the spinal cord having an anoxic core (Okada et al. 1993, Brockhaus et al. 1993). Wilson et al. (2003) showed that the neonatal mouse *in vitro* spinal cord preparation possesses an anoxic or hypoxic core during locomotor-like output. An anoxic core is worrisome to researchers because this is not a normal physiological condition. With the larger size of the neonatal rat spinal cord, the possibility of an anoxic core is even greater, however anoxia has not interfered with the reliability of the *in vitro* neonatal rat to produce locomotor-like output, (Nishimaru & Kudo 2000).

Pharmacologically evoked Locomotion in Animal Models

Glutamate and NMDA

Glutamate is an excitatory amino acid and can activate ion channels receptors defined by the specific ligands that bind to them. These include: the NMDA receptor, the kainate receptor, the AMPA receptor, and the L-AP4 receptor. It is important to note that these four receptors have subtypes. Glutamate can also activate several subtypes of metabotropic glutamate receptors.

The receptor/channel itself has oscillatory behaviour that can underlie its modulatory action on motoneurons. The NMDA receptor has voltage sensitivity (Nowak

et al. 1984), slow kinetics (Lester et al. 1990), and permeability to Ca^{2+} (Mayer et al. 1987). The neuro-toxin tetrotoxin (TTX) inhibits the Na^{+} current generating an action potential in neurons. When the Na^{+} action potential is blocked with application of TTX to the bath, addition of NMDA results in a rhythmic fluctuation in the membrane potential of motoneurons (Hochman et al.1994).

The NMDA receptor is involved in locomotion and intrathecal injection of NMDA to the lumbar segments in the cat spinal cord initiates locomotor activity (Douglas et al. 1993). Dale & Roberts (1984, 1985) showed that the slow kinetics of the NMDA receptor contributed to motor output in Xenopus embryo. Grillner & Wallen (1985) showed that the permeability of the receptor to Ca^{2+} and its voltage dependant block by Mg^{2+} are vital to the generation of pacemaker-like oscillations in neurons in the Lamprey. In the *in vitro* neonatal rat spinal cord, NMDA receptor activation occurs during fictive locomotion (Smith et al. 1987) and is able to induce a locomotor-like pattern of activity seen in ventral root recordings (Kudo & Yamada 1987; Smith & Feldman 1987, Cazalets et al. 1990). It has also been found that there was a critical concentration for NMDA (10-15μM) required to evoke activity and that the frequency of bursting increased by increasing concentration, (Smith et al. 1987, Kudo & Yamada 1987).

The effects of kainate and glutamate on the generation of a rhythmic activity have been tested. Kudo and Yamada found that kainate (2-10 μM) and glutamate (100-500 μM) evoked tonic VR activity (Kudo & Yamada 1987). Cowley & Schmidt (1994b) examined the ability of NMDA to generate rhythmic ENG activity, they demonstrated that 83% of the time NMDA induced rhythmic ENG activity, but in only 2/17 cases was

this activity reminiscent of locomotion. Hochman et al. (1994) have shown that NMDA receptor activation caused voltage oscillations in neonatal rat motoneurons. TTX application showed that the voltage oscillations were not mediated by synaptic connections, but were mediated intrinsically by the neurons themselves. Further MacLean (1998) found that NMDA could generate plateau potentials in motoneurons.

As mentioned NMDA has modulatory action on motoneurons that can cause rhythmic depolarization, and can generate plateau potentials which underlie bistability. NMDA has the ability to increase excitability in motoneurons and make motoneuron membrane voltages oscillate, perhaps contributing to some of the rhythmicity that is needed for locomotion. The accumulating evidence in the literature supports the idea that NMDA plays a large role in the initiation and maintenance of locomotion.

Acetylcholine

Acetylcholine (Ach) has been reported to induce locomotor-like rhythmicity in the *in vitro* neonatal rat spinal cord (Atsuta et al. 1991, Smith et al. 1988). However when examined with recordings from flexor and extensor nerves Cowley & Schmidt (1994b) showed that their activity was synchronous in flexors and extensors, not alternating. This has lead researchers to conclude that Ach may not induce locomotion on its own.

Dopamine/NA

Mammalian spinal cords contain descending axons and terminals of supra-spinal 5-HT or serotonergic, noradrenergic (NA), and dopaminergic neurons (Grillner 1975). In the *in vitro* neonatal rat preparation dopamine has been shown to induce a locomotor pattern that was slow and had long-lasting alternation (Jankowska et al. 1967, Atsuta et al. 1991, Kiehn & Kjaerulff, 1996). It has recently been found that the combination of

dopamine with NMDA and 5-HT can produce a robust locomotor-like rhythm in the neonatal mouse (Jiang et al. 1999, Whelan et al. 2000).

Less is known about the actions of NA on spinal networks but it is known that it can generate locomotor activity in the cat (Barbeau & Rossignol 1991, Kiehn et al. 1992). The ability of NA to generate a locomotor-like output was examined by Sillar et al. (1994), in the *in vitro* neonatal rat preparation. They found that NA induced irregular tonic discharges or an irregular slow rhythm, though in a few cases NA induced a slow locomotor-like rhythm. Kiehn et al. (1999) found that when applied during ongoing NMDA/5-HT induced locomotor activity, NA slowed the rhythm and could reinforce rhythmic locomotor activity.

5-HT

For a comprehensive review of the development and anatomy of 5-HT and other descending systems in the neonatal rat spinal cord, see Lakke (1997). Most 5-HT in the rat spinal cord originates from cells located in the medullary raphe pallidus, raphe obscuris, and raphe magnus (Dahlstrom & Fuxe 1964). The terminals from these cells can be found at all spinal cord levels, and a single raphe neuron can send axon collaterals to both the cervical and lumbar cord. Rat raphe neurons are generated between embryonic days 11 and 15 (Kudo et al. 1993). The axons of raphe neurons enter the cervical cord, via the ventral and lateral funiculi, at embryonic day 13-14 and reach the lumbar cord by E15-16. Postnatal day 3 the 5-HT innervation is organized into zones involving the motoneuron area in the ventral horn of the cervical and lumbar segments and intermediolateral column of the thoracic cord (Rajaofetra et al. 1987). By postnatal day 9 the innervation closely resembles the organization seen in the adult (Rajaofetra et

al. 1987). Research has demonstrated that at embryonic day 18-21, motoneurons are depolarized in response to 5-HT_{1A}, 5-HT_2, and 5-HT_3 receptor agonists (Ziskind-Conhaim et al. 1993, Takahashi & Berger 1990).

Labeling studies have found 5-HT at every level of the spinal cord and in every laminae that the receptor subtypes are much more restricted. For example 5-HT_1 receptors are found in the dorsal horn while 5-HT_2 receptors a found mainly in the ventral horn (Fischette et al. 1987). The 5-HT_{2A} receptor is found at high levels in the ventral horn, dorsal root afferents and dorsal root ganglia (Cornea-Hebert et al. 1999). 5-HT_{2c} receptors are found throughout the grey matter of the spinal cord, also on presynaptic on the nerve terminals of descending 5-HT fibers in the dorsal thoracic cord, and may function as autoreceptors (Brown et al. 1988). In the ventral horn 5-HT_{2C} receptors are located postsynaptically (Maeshima et al. 1998). 5-HT_3 receptors are present on both dorsal horn neurons and primary afferents where they may be involved in pain regulation. Fyda & Jordan (1997) have provided evidence that 5-HT_7 receptors are an important part of the locomotor process.

In the neonatal rat spinal cord 5-HT_2 receptor activation causes a depolarization of motoneurons via a decreased K^+ conductance, whereas 5-HT_{1A} receptor activation evokes a hyperpolarization by increased K^+ conductance (Elliott & Wallis 1990). Consistent with the idea that 5-HT_2 receptors increase excitation in the spinal cord, 5-HT_2 receptor activation restores extensor tone and stretch reflex excitability in cats after spinal cord transection (Miller et al. 1996), increases the excitability of motoneurons (Yamakazi et al. 1992), facilitates polysynaptic reflex transmission in the rat (Nagano et al. 1988).

5-HT is known to initiate and/or modulate locomotor output in many vertebrates including: amphibian tadpoles (Sillar et al. 1994, Woolston et al. 1994), lampreys (Christenson et al. 1989), neonatal rats (Sqalli-Housanini et al. 1993; Kjaerrulff & Kiehn 1996), and cats (Barbeau & Rossignol 1991). In chronic spinal cats, 5-hydroxytryptophan (5-HTP), a precursor of 5-HT, administered intravenously and alone, increased the tonic level of excitation in motoneurons but did not induce locomotion (Barbeau & Rossignol 1991). When 5-HTP was administered with clonidine (a noradrenergic agonist), a robust locomotor pattern was produced (Viala & Buser, 1971, Barbeau & Rossignol 1991). In the *in vitro* neonatal rat preparation 5-HT alone is able to induce a locomotor pattern (Cazalets et al. 1990, Cazalet 1992, Cowley & Schmidt 1994b). Cowley & Schmidt (1994b) and Squalli-Houssani et al. (1993) showed that administration of 5-HT alone could produce a locomotor-like output more consistent than NMDA alone.

The addition of pharmacological agents to the bath to produce locomotion in *in vitro* preparations is an efficient way to evoke locomotion, it is not the ideal for the study of Vth hyperpolarization. It is known that 5-HT and NA themselves are capable of hyperpolarizing Vth (Fedirchuk & Dai 2004). Therefore if 5-HT were added to the bath to initiate locomotion an effect on Vth could not be ascribed solely to activity of the locomotor CPG. Therefore the present study used electrical stimulation of the brainstem to activate spinal motor systems and not the exogenous application of monoamines.

Activation of the Locomotor CPG by Electrical Stimulation

Research has shown that stimulation of many different areas of the brain, brainstem and spinal cord can produce locomotion in the cat (Jordan 1991, Mori 2001).

These include midbrain regions known as the MLR (Shik et al. 1966). Electrical stimulation of the MLR in paralyzed decerebrate cats produces a centrally-generated pattern of locomotor output similar to normal locomotion in adult cats (Rossignol 1996). The animal is held in a stereotaxic frame and electrical stimulation of the MLR produces a motor output comparable to overground locomotion. Paralysis of the animal allows intracellular recordings from spinal neurons and electroneurographic (ENG) activity that closely resembles locomotion without movement (i.e. fictive locomotion). This fictive locomotion preparation has produced most of the literature about locomotion and spinal reflexes in mammals.

The MLR pathway originates in the cuneiform nucleus in the rostral pons, contacts the medial reticular formation (MRF) in the lower brainstem, and descends to the lumbar spinal cord in the VLF. The VLF is considered an essential descending pathway for the initiation of locomotion, as lesioning the VLF abolished MLR-evoked locomotion in the cat (Steeves & Jordan 1980; Shefchyk et al. 1984; Noga et al. 1991). The MLR for the adult rat has been found to exist in the same brainstem area as the cat (Coles et al. 1989).

Anterograde labeling studies by Garcia-Rill et al. (1983) have shown that the MLR projects to the dorsolateral pons and medulla, and the medioventral medulla (MED). Evidence the MED is important for locomotion was supported by Shefchyk et al. (1984), who found that cooling the MED resulted in blockade of MLR-induced locomotion in the cat. Kinjo et al. (1989) has also described the ability of electrical stimulation of the MED to produce locomotion in the adult rat.

Electrically Evoked Locomotion in the Neonatal Rat

In 1988 Atsuta et al. developed a neonatal rat brainstem-spinal cord preparation capable of electrically-induced locomotion. They found that low-frequency stimulation (3-6 Hz) of short duration (0.1-0.3 ms) pulses was able to induce air stepping and EMG output representative of a locomotor pattern. They found that the most effective site for eliciting locomotion was located in the MED. In an effort to prove that the electrical stimulation was evoking a physiological activation of the brainstem and spinal cord glycine was added to the cervical bath. Glycine blocked the electrically-evoked locomotion and indicated that descending or propriospinal systems are responsible for physiological activation of the lumbar region to produce locomotion (Atsuta et al. 1988). The behaviour was similar to true locomotion in that the EMGs of electrically induced locomotion in neonate and adult rats were similar (Atsuta et al. 1990).

Magnuson et al. (1995) demonstrated the ability of short trains of electrical stimulation to the VLF to produce locomotor-like output in the neonatal rat. Magnuson & Trinder (1997) found that a L2/L3 midsagittal lesion could abolish VLF stimulation-evoked locomotor-like activity. However they found that NMDA (2-5 μM) and 5-HT (20-50 μM) could still induce rhythmic locomotor output in the lesioned spinal cord. It was concluded that the VLF is an important locomotor command pathway in the neonatal rat. They also stated that the pathway has a bilateral projection in the lower thoracic and upper lumbar segments, demonstrated by the abolition of locomotor-like activity by a midsagittal lesion.

The electrical stimulation of the neonatal ventromedial medulla (VMM) has recently been proven to be a useful tool to examine locomotion and the release of endogenous 5-HT, DA, and NA during locomotion in the neonatal rat (Fyda & Jordan

1997). Fyda & Jordan electrically stimulated an area of the VMM and found that up to 400 pg of NE, 250 pg of 5-HT, and 20 pg of Dopa are released into the lumbar cord during 30 minutes of locomotion, while during control periods (no locomotion) monoamines were not detected. Fyda & Jordan found that that application of ketanserin (20 μM) and clozapine (0.25 μM) 5-HT_{2A} and 5-HT_7 receptor antagonists respectively blocked electrically-induced locomotion. They concluded that for electrically-stimulated locomotion, the responsive elements of the CPG are located from T10 to L2 segments of the spinal cord and the CPG is responsive to both 5-HT_7 and 5-HT_{2A} receptor activation. Researchers have shown that the neonatal rat spinal cord could produce locomotor-like output from trains of dorsal root stimuli, (Budakova 1972, Smith et al. 1988, Marchetti et al., 2001). The type of locomotor output was consistent with the fictive locomotor-like output produced from bath application of NMDA, 5-HT, or high K^+. Marchetti suggested that they activated the CPG by stimulating dorsal afferents, which project to the CPG itself (Hultborn et al. 1998).

Recently Zaporozhets et al. (2004) described a reliable technique for the induction of locomotor-like activity in the in vitro neonatal rat spinal cord using electrical stimulation of the brainstem. Contrasting the focused stimulation of specific brainstem structures employed by Atsuta et al.(1988) or Fyda & Jordan (1997), Zaporozhets et al. used macroelectrical stimulation of the brainstem to evoke locomotor-like activity. They found that macrostimulation produced locomotor-like activity similar in frequency reported in other preparations (Atsuta et al. 1988, Fyda & Jordan 1997).

Cellular properties can be altered during different behaviours

Krawitz et al. (2001) described the state-dependant hyperpolarization of Vth

during fictive locomotion in the cat. Other animal models have shown altered cellular properties during different behaviours. Aplysia and crustaceans are invertebrate animal models that have been used to study neural networks. Ionic mechanisms underlying bursting have been studied in the R15 neuron in Aplysia (Adams & Benson 1985). Dopa and 5-HT are both able to decrease the ionic currents that generate bursts in these pacemaker neurons (Harris-Warrick & Marder 1991). The crustacean Somatogastric Ganglion (STG) is a population of cells that are active during feeding. In the STG some neurons are conditional bursters in that they only burst when influenced by modulatory agents. For example, the AB neuron of crustacean STG is a conditional bursting neuron that is activated by 5-HT and Dopa (Harris-Warrick & Marder 1991). Different ionic mechanisms underlie amine induced bursting in this neuron, Dopa requires Ca^{2+} currents while 5-HT generates bursting via a TTX-sensitive Na^{+} current (Harris-Warrick and Flamm 1987).

An interesting aspect of neuronal physiology is the ability of some neurons to display bistable membrane plateau potentials. These types of neurons have the ability to exist at different stable membrane potentials. These include a resting potential and a depolarized state of tonic firing. The general opinion within the literature is that plateau potentials reduce the need for steady ongoing synaptic drive during maintained activity and produce a more steady and predicable firing (Kiehn & Eken 1998). Plateau potentials have been found in turtle (Hounsgaard & Kiehn 1989) and the cat (Conway et al. 1988, Hounsgaard et al. 1988, Paroschy 2000). The neurotransmitter 5-HT is known to facilitate plateau potentials by decreasing the resting K^{+} current that in turn increases the opening time of the L-type Ca^{2+} channels (Hounsgaard et al. 1989). Hounsgaard et al.

(1989) also found that 5-HT reduces the Ca^{2+} activated K^{+} current needed for the slow spike afterhyperpolarization. Alaburda & Hounsgaard (2003) have found in the turtle that during a behaviour resembling scratch-like episodes, the excitability increased in most rhythmic motoneurons via a facilitation of the Ca^{+2} -channel mediating the persistent inward Ca^{+2} current underlying plateau potentials (Alaburda & Hounsgaard 2003).

It is clear from lower animal preparations that during different behaviours there is modification of motoneuron properties by endogenous neurotransmitter systems acting as modulatory systems. The generation of locomotion in lamprey is initiated by the brainstem and mediated by glutamatergic excitation of the spinal circuitry from reticulospinal neurons (Ohta & Grillner 1989). In the lamprey spinal cord, activation of NMDA receptors induces plateau potentials in motoneurons and interneurons (Alford & Sigvardt 1989) and facilitates neuronal bursting (Grillner et al. 1991) properties thought to be important for swimming in the lamprey. Therefore modification of motoneuron properties during behaviors appears to be a basic mechanism conserved across species.

Threshold properties

An important property of the action potential is its initiation at a particular membrane potential, called voltage threshold. Indeed, action potentials never occur without a depolarizing stimulus that brings the membrane to this level. The depolarizing "trigger" can be one of several events: a synaptic input, a receptor potential generated by specialized receptor organs, the endogenous pacemaker activity of cells that generate action potentials spontaneously, or the local current that mediates the spread of the action potential down the axon.

If there is a subthreshold depolarization, the rate of increased Na^+ entry is less than the rate of K^+ exit, and the cell will return to resting membrane potential. A more precise definition of threshold, is that value of membrane potential, at which the current carried by Na^+ entering the neuron is greater than the K^+ current that is flowing out. Once the triggering event depolarizes the membrane beyond this point, the positive feedback loop of Na^+ entry fires an action potential. Underlying the propagated action potential is a regenerative wave of opening and closing of voltage-gated Na^+ ion channels that sweeps along the axon. When Na^+ channels open, Na^+ ions enters the axon, driven by the Na^+ concentration and voltage gradient (Hodgkin & Huxley 1952). This system positively feeds back on itself to completely depolarize the cell and it will come very close to having a membrane potential close to the Na^+ equalibrium potential (Coombs et al. 1957).

Threshold is an active property of all neurons and based on channel kinetics, distribution, and density. Threshold can vary from cell to cell because of these factors. However it had been thought since the two channels underlying the action potential (the voltage gated Na^+ channel and voltage gated K^+ channel) were activated at precise voltages that the voltage threshold for every cell remained constant and only channel availability could be modulated, thereby leading to the idea of voltage threshold (Gustafsson & Pinter 1984). However there is growing evidence that the channel kinetics and voltage sensitivity can be modulated (see below).

Neuronal Properties are Dynamic and Altered During Locomotion

Reduced Afterhyperpolarization

Brownstone et al. (1992) found that motoneurons had an after hyperpolarization (AHP) trajectory that was depressed during locomotion compared to AHPs observed

during repetitive firing at similar rates, induced by the injection of depolarizing current through a microelectrode. The decreased amplitude could be due to a decrease in AHP conductance. The reduction in AHP was also seen during locomotor-like activity in the neonatal rat spinal cord preparation (Schmidt 1994). Because this preparation is without the influence of supraspinal structures, Schmidt (1994) concluded that the spinal cord locomotor circuitry is able to modify motoneuron properties and depress the AHP.

Motoneuron Firing in Response to Current Injection During Fictive Locomotion

Brownstone et al. (1992) examined the frequency-current relation of motoneurons in the presence and absence of fictive locomotion. They found that there was no relation between the frequency of firing and the current injected during fictive locomotion. Thus, motoneurons did not fire faster with more current injection. However, Brownstone et al. (1992) reported that less current needed to be injected during locomotion to make the cell fire even though motoneurons did not fire faster with more current once they were already firing repetitively. Different explanations might account for this: first, action potentials might be fired on top of plateau potentials; second is the possibility that motoneurons receive large synaptic input which lead to the action potentials. Brownstone et al. (1992) concluded repetitive firing in motoneurones during fictive locomotion is not produced by somatic depolarisation alone, and that motoneurones do not behave as simple input-output devices during locomotion

Motoneuron Locomotor Drive Potential Amplitudes Increase With Depolarization

Brownstone et al. (1994) examined the voltage dependance of excitatory synaptic events during fictive locomotion in the cat. They found that during fictive locomotion, a ramp of depolarizing current injected into a motoneuron caused a nonlinear increase in

the excitation that is seen as a jump in the amplitude of the locomotor drive potential (LDP). In attempting to determine the role of intrinsic membrane properties in the voltage-dependant excitation, ramp currents were injected in the absence of locomotion to see if plateau potentials could be evoked. Brownstone et al. (1994) found that in six motoneurons intracellular injected current did cause membrane potential to jump to a plateau potential. They hypothesized therefore that plateau potentials could increase the amplitude of LDPs.

Vth Hyperpolarizes During Fictive Locomotion

Recently Krawitz et al. (2001) described a state-dependant hyperpolarization of action potential Vth in cat motoneurons. They found the Vth of motoneurons hyperpolarized during fictive locomotion. Further they found that Vth hyperpolarized for all motoneurons tested, whether they were flexor or extensors, or of high or low rheobase. They also found that the Vth hyperpolarization was present in both phases of the step cycle and was not dependant on repetitive firing. Motoneurons were found to recover within seconds after the MLR stimulation was removed. The Vth did not vary rhythmically with membrane potential fluctuations during locomotion and was reduced to the same extent for spikes produced from the locomotor circuitry and intracellular current injection. It is important to note that Vth hyperpolarization was not accompanied by a consistent change in the amplitude or duration of the action potential, suggesting the mechanism(s) responsible for modulation of Vth do not involve a large increase in the amplitude of the sodium conductance underlying firing (see Dai et al. 2002).

The Membrane Potential for activating Action Potentials is not a Fixed Value

Vth tends to be Higher in Higher Rheobase Neurons

There are three types of motor units referred to in the literature: fast fatigable (FF), fast-fatigue resistant (FR), and slow (S). Gustafsson & Pinter (1984) tried to classify the motoneurons that innervate these three motor unit types into groups by using rheobase currents. When Gustafsson & Pinter (1984) examined the Vth for the three motoneuron groups they found that the group with the lowest rheobase current also had the lowest Vth, independent of resting membrane potential.

Depolarization of Vth Occurs During a Train of Repetitive Firing

Work done in cats by Kolmodin & Skoglund (1958) found that in a single motoneuron Vth was not a fixed value. They found that an action potential occurring later in a train of action potentials occurred at a more depolarized membrane potential than those occurring initially. Kolmodin & Skoglund (1958) found that the firing level in a single neuron could show considerable fluctuations at a given activity level (e.g. initial Vth of -55 mV was increased to a Vth of –48 mV near the end of the train), and that the spike produced from current injection was elicited at a more depolarized membrane potential than during natural activation (-62mV and -58mV, respectively).

Motoneuron Threshold Potential is Depolarized in Chronic Spinal Animals

Hochman & McCrea (1994) examined the properties of motoneurons from 6-week chronic spinal cats. The found that threshold for action potentials were initiated at a higher membrane voltage (i.e. more depolarized) in the chronic spinal animal. They concluded that after spinalization there was an alteration in the subthreshold properties that determined Vth.

5-HT, NA and Ach are able to Hyperpolarize Vth

In addition to the ability of 5-HT to induce locomotor activity in animal models, 5-HT can depolarize motoneurons (Neuman 1985, Connell & Wallis 1988, Elliott & Wallis 1990, Takahashi & Berger 1990, Binder et al. 1993) by: 1) the enhancement of a slow depolarizing inward rectifier current of K^+ and Na^+ ions (Wang & Dun 1990, Takahashi & Berger 1990, Kjaerulff & Kiehn 2001), 2) the facilitation of a low voltage-activated Ca^{2+} current (Berger & Takahashi 1990), and 3) the inhibition of a fast inward rectifier current carried by K^+ currents (I_{Kir}), (Kjaerulff & Kiehn 2001). These effects reduce leak current out of the cell, thereby enhancing neuronal excitability. 5-HT can reduce the AHP following an action potential (Vandongen et al. 1986) and can also facilitate non-linear integrative properties in motoneurons. 5-HT promotes bistable firing in spinal animals (Housngaard et al. 1988; Hounsgaard & Kiehn 1989) and monoaminergic agents enhance persistent inward currents (Lee & Heckman 1999). Also, 5-HT facilitates conductances mediated by NMDA receptors (MacLean et al. 1998, MacLean & Schmidt 2001). Recently Fedirchuk & Dai (2004) demonstrated the ability of 5-HT and NA to hyperpolarize Vth of lumbar motoneurons in the hemi-sected neonatal rat spinal cord.

It is clear that the cat model has provided excellent locomotion and allowed the discovery of locomotor-related Vth hyperpolarization, but there is a need to understand the underlying mechanisms. To this end a preparation that could provide locomotion or locomotor-like behaviour evoked by electrical stimulation and yet be amenable to pharmacological intervention would be desirable. In addition an understanding of the mechanisms behind Vth hyperpolarization would be aided by a preparation where motoneuron membrane properties are potentially modifiable; and neurochemical agents

or receptor antagonists could be quickly added and removed, would be ideal. For these reasons the *in vitro* neonatal rat brainstem/spinal cord preparation was chosen for experimental design.

Hypothesis

Neonatal rat motoneurons undergo hyperpolarization of Vth during locomotor-like activity.

Specific Objectives

1) Establish a neonatal rat brainstem/ spinal cord preparation in the laboratory where electrical brainstem stimulation can produce locomotor-like activity.
2) Use whole-cell patch clamp techniques to demonstrate Vth hyperpolarization of motoneurons occurs in the neonatal rat during electrically evoked locomotion.
3) Determine if changes in Vth are dependant on the locomotor state.
4) Assess whether electrical stimulation of dorsal roots can also hyperpolarize Vth.
5) Assess the ability of 5-HT receptor antagonists to block the brainstem-evoked change in Vth.

Methods

Neonatal rat brainstem-spinal cord preparations aged 1-5 days were included in this study. All animal protocols were developed in accordance with Canadian Council on Animal Care guidelines and were approved by the University of Manitoba animal protocol committee. The animals were anesthetized with halothane in a sealed chamber. Once anesthetized, the animals were moved and pinned to a slygard-bottomed dish containing 1°C aCSF. A precollicular/postmammillary mechanical decerebration was rapidly performed, and the thorax eviscerated, the preparation was then transferred to a clean slygard-bottomed chamber. The animal was super fused in this chamber in 4°C aCSF: 125 NaCL, 2.5 KCL, 1 MgCl2, 1.25 Na2HPO4, 26 NaHCo3, 2.0 CaCl2, 25 D-Glucose (all values in mM).

The aCSF was continuously aerated with 95%O_2 / 5% CO_2. A dorsal and ventral laminectomy was performed to expose the spinal cord and brainstem. Using fine forceps the medulla and spinal dura matter were removed. All spinal roots were cut near the ganglia and the spinal cord brainstem was transferred to clean sylgard recording chamber. The preparation was pinned ventral side up with 0.1 mm insect pins. In some experiments a midsagittal section of the spinal cord was performed between L3 and S2, allowing microelectrode access to the ventral horn neurons. The preparation was allowed 30 minutes of recovery time before experimenting began. This time allowed the preparation to slowly warm to 25 °C. Temperature was maintained with the use of a dissecting light, and monitored with a temperature probe in the bath. The temperature remained constant at 25 °C during all experiments, except where noted.

Stimulation and Recording

Locomotor rhythmic activity was evoked through electrical stimulation of the VMM. A monopolar tungsten electrode (10 –150 KΩ) (Microprobe, Inc. 11715 Tifton Drive Potomac, MD 20854) was lowered onto the surface of the ventral medulla. The electrode was positioned: AP: 1.0-2.0 mm caudal to the level of transection, ML: 1.5-2.0 mm from midline, DV: 0.0 mm from ventral surface (Figure 3). The optimal site for evoking rhythmic activity was determined by repositioning the stimulating electrode at 0.25 mm increments mediolaterally until stable rhythmic activity was obtained. Electrical stimulation using 200 μA – 10mA, 5 ms duration square pulses at 1-3 Hz was used.

Ventral root recordings were obtained using plastic suction electrodes. Ventral root recordings from ipsilateral L2 (iL2) and iL5, as well as contralateral L2 (cL2) and cL5 were used to monitor motor output (Figure 3). These ventral roots were monitored because L2 largely correlates with flexor bursting and L5 correlates with extensor bursting (Cowley & Schmidt 1994a, Kiehn an Kjaerulff 1996). While Cowley & Schmidt (1994a) have pointed out ENG activity is more accurate than ventral root recordings for the definition of locomotor-like activity, the mechanical stability for intracellular recordings in the current study required that the hind limbs be removed before the experiment began. Ventral root recordings were digitized (5000Hz), stored and analyzed using software (Capture program) developed by the SCRC.

Intracellular Recording

Intracellular voltage clamp recordings were made to determine changes in voltage threshold. Glass patch clamp electrodes (4.1 - 4.5 MΩ) (containing: K-gluconate 140μM, EGTA 0.2μM, Hepes 10 μM, KOH 35 μM) were used to blind patch neurons

and motoneurons. Motoneuron identity was determined by antidromic stimulation of the nearby ventral root (Figure 3). Intracellular recordings were amplified using an Axon Instruments Patch Clamp Amplifier 1-D in voltage clamp mode. All intracellular recordings were performed using Axon instruments software p-clamp 7.0. Individual motoneurons were clamped at –60 mV and a protocol used to step the holding potential up by +2 mV every sweep. Protocol for the p-clamp recording was 20 sweeps with each sweep lasting 514 ms. The depolarized phase lasted 100 ms. Baseline Vth was defined as the level of the depolarizing step that produced the first fast inward current. This level was then used as a reference for Vth determination following experimental manipulation.

In this study voltage steps were given using 2mV increments. While this only allowed detection of Vth changes >2 mV larger steps the exploration of significant changes in Vth. Figure 14 demonstrates the stability of a –2mv change in threshold. This neuron was recorded for over 15 minutes and a 2mV change was reproducibly recorded with stimulation of the VMM. Further the 2mv change could be abolished and recovered. Therefore we considered 2 mV changes in Vth to be significant changes in Vth and not random fluctuations in Vth of the neuron.

Antidromic Stimulation

Electrical stimulation of the ventral root produces an antidromically propagating action potential that is recordable in the motoneuron. A ventral root suction electrode that could be switched from record to stimulate was used, allowing the same electrode to provide different functions. Stimulation parameters ranged from 300 – 600 μA, 1ms 1Hz. Testing for antidromic activation could not be done in all experiments, and it is possible that in some preparations where it was attempted the ventral root fibers may

have been damaged by the position of the patch pipette. Therefore it is likely that there are motoneurons included in our sample of "unidentified" ventral horn neurons.

Cooled Cervical Partition

In some experiments the cervical segments were partitioned using 35 mm film and Vaseline (Figure 3), creating individual brainstem, cervical and lumbar baths. The cervical partition was cooled to 4 °C by washing the partition with cooled aCSF. The lumbar partition was monitored and kept at 25 °C by washing with 25°C aCSF.

Dorsal Root Stimulation

In some experiments dorsal roots were stimulated using the constant current stimulator. Stimulation parameters ranged between 100- 600 μA, 5ms, 1-3 Hz. Plastic suction electrodes were attached to the dorsal roots. This allowed an alternate means of evoking ventral root output and assessment of Vth.

Antagonists

In some experiments, antagonists of 5-HT were applied to the bath to determine the pharmacology underlying the hyperpolarization of Vth. Ketanserin (2-20 uM) was added to the bath and allowed to diffuse into the cord. As an antagonist Ketanserin has the greatest affinity for the the 5-HT_{2A} receptor with an affinity of 1nM. Ketanserin has affinity for other 5-HT receptor systems, 5-HT_{1A} (>1000 nM), 5-HT_{1B} (1910 nM), 5-HT_{2c} (50nM), 5-HT_4 (13 nM), 5-HT_6 (>10 000 nM), 5-HT_7 (43 nM) (Cope 2001). Stimulation of the brainstem at the same level as control using the same voltage clamp protocol allowed the assessment of Vth during brainstem stimulation, but in the presence of the 5-HT_2 antagonist ketanserin.

Washout

Washout was accomplished by the suction removal of aCSF in the bath and the immediate replacement by clean, room temperature aCSF bubbled with 95% O_2 / 5% CO_2. This was repeated 3 times over 5 minutes to ensure that pharmacological agents were removed from the tissue and bath.

Current Clamp

Current clamp recordings were done in some experiments by using the axopatch 1-D in current clamp mode. This allowed the recording of Vm during control and during electrical brainstem stimulation and as well as recordings of locomotor drive potentials (LDPs) during brainstem stimulation. These recordings were used to determine that the motoneuron being recorded from was recruited in the locomotor-like output. However this protocol was not used for Vth analysis, as the Axopatch 1-D cannot faithfully record action potentials in current clamp mode (Magistretti et al. 1996).

Notes on Development of Methods

The *in vitro* neonatal rat preparation used in this study was similar to other researchers' methodologies in extracting the brainstem-spinal cord from the body of the animal (Smith & Feldman 1987). One exception was that in addition to all aCSF being aerated with 95% O_2 / 5%Co_2 during the laminectomies, the aCSF that superfused was chilled to 4°C and was pressurized to 100kpa with 95% O_2 / 5%Co_2. The cooled and pressurized aCSF resulted in 5 times the O_2 being dissolved into the aCSF compared to room temperature bubbled aCSF (Table 1). This resulted in more O_2 being available to the tissue and may have increased brainstem tissue survival. Experiments using an O_2 meter in the dish showed that O_2 levels of the initially pressurized fluid remained well

above bubbled levels for up to 20 minutes. It is known that the decreased temperature of the tissue results in a lower metabolic rate and demand for oxygen (Bigelow 1958). In fact merely decreasing the temperature of the animals from 36 to 30 °C results in 50% less demand for oxygen (Bigelow 1958). Therefore these researchers maintain that cooling the tissue to 4°C increased its survival during the time from when the animal was eviscerated until adequate diffusion from direct contact with the oxygenated aCSF was established.

Atusuta et al, (1988) and Fyda & Jordan (1997) have demonstrated that they were able to produce a locomotor-like output from the neonatal rat brainstem-spinal cord by electrically stimulating the VMM. They reported stimulus intensities of 100-250 μA, 5ms, 1-3 Hz. However attempts to replicate the above results using their stimulation parameters were unsuccessful. The method of electrical stimulation of the brainstem similar to Zaporozhets et al. (2004) was employed to evoke locomotor-like activity. Methods described by Zaporozhets et al. (2004) used substantially more current (500 μA - 10 mA) than previous researchers (Atsuta et al. 1988, Fyda & Jordan 1997), Proper grounding was needed to control volume conduction. It was found in the present study that poor grounding resulted in fluctuations in leak current of the intracellular electrode and large stimulation artifacts when stimulating. However using chlorided grounds with optimal placement of the ground in the bath, the above problems with intracellular recording were eliminated.

Plastic suction electrodes were developed to record from ventral roots. The standard for neonatal ventral root recordings is glass suction electrodes. Plastic suction electrodes were pulled from 1cc plastic syringes, heated over a flame. The syringe pulled

into a very fine tube; this was cut with dissecting scissors to the desired size (60 – 100uM).

Portions of this data have previously been presented (Gilmore and Fedirchuk 2002, Gilmore and Fedirchuk 2004).

Results

Electrical Stimulation of the Brainstem Can Produce a Locomotor-like Output

As reported elsewhere locomotor-like activity could be evoked from brainstem stimulation (Atsuta et al. 1988, Fyda & Jordan 1997, Zaporozhets et al. 2004). The locomotor-like activity typically occurred with a frequency of 0.3Hz – 0.5 Hz. This frequency of activity is consistent with frequencies reported by Fyda & Jordan, and during chemically evoked locomotor-like activity (Atsuta et al., 1988, Cowley & Schmidt 1994b, Cazalets 1992, Zaporozhets et al. 2004).

Electrical stimulation of the neonatal rat brainstem spinal produced a variety of VR motor outputs. In the present study the ventral root output was characterized as follows:

1. **Locomotor-like activity** was defined as alternation between the ipsilateral and contralateral second lumbar root (lL2 and rL2), and alternation between at least one lumbar root 5 (lL5 and rL5) on the same side (Figure 4 is an example of locomotor-like activity).
2. **Ipsislateral/Contralateral activity (Ipsi/contra)** was alternation between the L2 and the contralateral L2. Ipsi/contra activity is alternating rhythmic activity but is considered a different motor output compared to locomotor-like.
3. **Ipsilateral activity** was alternation in a L2 root and the ipsilateral L5 that was alternating.
4. **Rhythmic activity** was bursting activity that did not alternate with other roots. It could also be a single root rhythmically discharging.

5. **Tonic activity** was activity where there was no bursting but increased tonic output from one or more roots.
6. **No ventral root output** was when no measurable activity was elicited from the ventral roots by the brainstem stimulation.

Figure (4A) shows an example of locomotor-like ventral root output. Stimulation of the VMM was delivered for the entire 140 seconds (1mA, 1 ms, and 1Hz) with locomotor-like activity persisting throughout this period. Ventral root recordings are from the rL2, lL2 and lL5. In Figure 4A there is alternation between the rL2 and lL2 roots, the lL5 root is alternating with the lL2 root. This example is comparable to the locomotor-like activity produced by pharmacological agents (Cowley & Schmidt 1994a, Zaporozhets et al. 2004).

Electrical Stimulation of the Brainstem Activates Brainstem Systems

We considered whether current spread might have caused a direct activation of spinal circuitry and initiated locomotion. In order to be certain that the brainstem systems were mediating the locomotor-like activity, cervical spinal segments were cooled to 4 ° C by selectively cooling the cervical partition (n= 6). Cooling the neural tissue to 4 ° C blocks action potential propagation (Castro-Moure & Goshgarian 1996). Figure 4B shows that cooling the cervical spinal cord to 4 ° C blocks ventral root activity produced by VMM simulation. Notice that there are stimulus artifacts in ventral root recordings. No ventral root output was elicited in either the lL2 or rL2 roots or in the lL5. The lumbar spinal cord was kept at 25° C by wash-out of the lumbar partition with warm

aCSF (25 ° C). Figure 4C shows re-occurrence of locomotor-like activity 10 minutes after cervical partition was rewarmed to 25° C.

Effect of VMM stimulation on Motoneurons and Unidentified Neuron Action Potentials

This study includes combined recordings of ventral roots with whole-cell voltage clamp recordings from motoneurons and unidentified neurons from within the lumbar segments of the neonatal rat spinal cord. It was found that for all antidromically identified motoneurons (n=36) recorded, there was no change in the peak amplitude (Paired t-test, p=0.688) or duration (Paired t-test, p= 0.185) of the first fast inward current with electrical stimulation of the VMM. In unidentified neurons (n=35), there was an increase in the peak amplitude that was significant during electrical stimulation of the VMM (Signed Rank Test, p=0.007), but there was no change in duration of the current (Signed Rank Test, p=0.058). The change in peak amplitude was skewed by three neurons in which the change in peak was decreased by >50% with stimulation of the VMM. If these three neurons were removed from the sample there was no change in peak or duration for the remaining unidentified neurons. However these three neurons are left in the sample because there was no criterion for their exclusion.

Electrical Stimulation of the Brainstem Produces a Hyperpolarization of Vth in Antidromically Identified Motoneurons

Figure 5A shows a voltage clamp recording of the first fast inward current from an antidromically identified motoneuron before and during brainstem stimulation. Figure 5A illustrates control before the stimulation; starting at an initial holding potential of –60mV, depolarizing voltage command steps of 100 ms duration were applied. The amplitude of successive steps was increased in +2mV increments. Vth was defined as the

first depolarized holding potential that elicited a fast inward current in the neurons. In this motoneuron the first trace that elicited a fast inward current was –34mV, therefore the Vth for activating the first fast inward current in this motoneuron was -34 mV. Figure 5B shows a voltage command trace of the first fast inward current, during electrical stimulation of the VMM (10 mA 5ms, 2Hz). The Vth changed to –38mV, a –4mV hyperpolarization. Figure 5C shows the accompanying ventral root recording for the rL2 and lL2 roots and the rL5 and lL5 roots, this output is consistent with locomotor-like output. Electrical stimulation of the brainstem produced a locomotor output that was accompanied by a hyperpolarization of Vth (n = 11).

Vth Hyperpolarization During Locomotor-like Motor Output

Figure 6 shows a summary of the data obtained from 11 neurons (identified and unidentified combined) where the Vth of the first fast inward current was recorded during control conditions and during VMM stimulation that evoked locomotor-like VR output. Figure 6A shows the Vth of each neuron in the absence of brainstem stimulation (control) and during stimulation. In 10/11 cases there is hyperpolarization of the first fast inward current during stimulation. Figure 6B re-plots this data as the relative amount of Vth hyperpolarization compared to control. The range of Vth change ranged from 0 to -18 mV (mean –6 mV). The mean Vth during control =-32.9+/-5.61, and the mean Vth during stimulation producing a locomotor output was -38.7 +/-9.26. The hyperpolarization of Vth was statistically significant (paired t-test, p = 0.004). Figure 6 shows that the hyperpolarization of Vth of the first fast inward current of neurons (identified and unidentified) occurs during locomotor-like VR output.

Vth Hyperpolarization During Ipsi/Contra VR Activity

Figure 7 shows a Vth data from nine neurons during stimulation of the VMM that produced an alternation of the ipsi/contra L2 roots or L5 roots. In all cells the Vth hyperpolarized during the brainstem stimulation. Figure 7B re-plots this data as the range of the Vth hyperpolarization. In all cases there is hyperpolarization of Vth of the first fast inward current (range –4 mV to –20 mV), (mean –10 mV). The hyperpolarization of Vth that occurred during stimulation that produced ipsi/contra VR activity was statistically significant (paired t-test, $p < 0.001$).

Vth Hyperpolarization During Ipsi VR Activity

Figure 8 shows the Vth data from six neurons where the VMM. stimulation produced an alternation between the Ipsi L2 and L5 roots but no contra activity. Figure 8B shows the range of Vth hyperpolarizations was 0 to -10 mV (mean –4 mV). The amount of hyperpolarization with stimulation producing Ipsi VR activity was statistically significant (paired t-test, $p = 0.028$).

Vth Change During Rhythmic Activity in only one VR

Figure 9 shows a summary of the data from 13 neurons where VMM stimulation only produced rhythmic activity in one of the L2 or L5 roots. Figure 9B re-plots the data as a relative range of Vth hyperpolarization, range +2 to -14mV, mean –5mV). The amount of hyperpolarization with stimulation producing rhythmic VR activity was statistically significant (paired t-test, $p = 0.002$).

Vth Hyperpolarization During Tonic VR Activity

Figure 10 displays a summary of data collected from eight neurons where VMM stimulation produced tonic activity in any of the L2 or L5 roots. Figure 10B re-plots the data as the relative range of Vth hyperpolarization, range 0 to -6mV, (mean –2.5mV).

The amount of hyperpolarization with stimulation producing tonic VR activity was statistically significant (paired t-test, p= 0.011).

Vth Hyperpolarization During No Ventral Root Output

Figure 11 displays the Vth data collected from 46 neurons where the first fast inward current was recorded during control and during stimulation of the VMM. In these trials stimulation failed to produce any activity the L2 or L5 roots. Figure 11A show the Vth during control on the left and the same neurons Vth during stimulation on the right. Figure 11B re-plots the data as the relative range of Vth hyperpolarization, range +2 to -16mV (mean –6 mV). The amount of hyperpolarization with stimulation producing no VR activity was statistically significant (paired t-test, p <0.001).

Summary of Vth for All Cells Independent of Motor-Pattern

In 64/71 neurons Vth hyperpolarized upon electrical stimulation of the VMM. In 4/71 neurons Vth did not change with electrical stimulation of the brainstem and in 3/71 cases Vth depolarized with electrical stimulation. In antidromically identified motoneurons (n=36), Vth depolarized in one cell (+2 mV) , was undetermined in two and Vth hyperpolarized (-2 to-14mV) in 33 cells. For unidentified neurons (n=35), Vth depolarized (+2 mV) in 2 cells, was unchanged in two cells, and Vth hyperpolarized (-2 to –20 mV) in 31 cells. The locomotor-like motor output did not produce a greater change in Vth compared to other motor outputs. An analysis of variance comparing all VR outputs found that Vth hyperpolarization was not greater with any one VR motor output (one way ANOVA, p = 0.079). The Vth change produced with electrical stimulation of the brainstem was not significantly greater among motoneurons (n=35) compared to non-motoneurons (n=36) (Mann-Whitney rank sign test, p=0.053).

Vth Hyperpolarization In One Neuron With Different Brainstem Evoked Motor Outputs

Electrical stimulation of the VMM did not always produce consistent ventral root motor output throughout the recording period with repeated electrical stimulation. Figure 12 displays a summary of the relative Vth hyperpolarization of neurons recorded during different ventral root output evoked using the same stimulation parameters. Figure 12 shows the change in Vth ranges from +2 to -10 mV. In only six of the 16 cases is the amount of Vth hyperpolarization the same in both VR patterns. However the appearance of ventral root activity had no relationship to degree of Vth hyperpolarization (paired t-test, $p = 0.119$), and no particular motor-output is associated with a greater amount of Vth hyperpolarization.

Hyperpolarization of Vth is Produced at Stimulus Intensities Sub-Threshold for Rhythmic Motor-Output

Is hyperpolarization of Vth dependant on VMM stimulus intensity? In three motoneurons the intensity of electrical stimulation of the brainstem was increased with repeated trials and Vth determined in each trial. Figure 13 shows the ventral root recordings of lL2, rL2 and lL5, rL5 roots; below these recordings are the duration and intensity of stimulus. In this example the VMM was stimulated at 5 different stimulus intensities (100 μA, 200 μA, 300 μA, 400 μA, 500 μA) 5ms, 1Hz. Below the stimulus bars is the observed change in Vth of the first fast inward current in a motoneuron, during assessment using voltage clamp mode.

Figure 13 shows that stimulating at 100μA, 5ms, 1Hz, does not produce a change in Vth nor any ventral root output. However stimulation at 200 - 400 μA, 5ms, 1Hz,

produced a –2mV Vth hyperpolarization of the first fast inward current in the absence of any rhythmic output of the ventral roots. Stimulation at 500μA produced a –4mV change in Vth and also produced an alternating motor output in the rL2 and lL2 roots. Stimulation at higher intensity (600 μA) produced the same rhythmic motor output and the same amount of Vth hyperpolarization (not shown).

Figure 13 is important in that it shows that Vth can be hyperpolarized at stimulus intensities sub-threshold for rhythmic motor-output and shows that clear separation of Vth from VR activity can be demonstrated in this preparation.

Cooling the Cervical Cord Blocks Vth Hyperpolarization

Figure 14A shows a voltage clamp recording where electrical stimulation of the brainstem evoked a -2mV change in Vth. Figure 14A shows the voltage clamp recording during control of the first fast inward current (left traces) and the voltage clamp recording during electrical stimulation of the first fast inward current (right traces). Below the voltage clamp recordings are the ventral root recording of the two-second lumbar roots and the two fifth lumbar roots. Stimulation was delivered to the VMM at 600μA, 5ms, 1Hz and evoked rhythmic activity. Electrical stimulation produced a hyperpolarization of Vth of the first fast inward current because during stimulation the second trace of the first fast inward current is activated at –38 mV as opposed to –36 mV compared to control.

Figure 14B shows that cooling the cervical partition to 4 ° C blocks the change in Vth and the ventral root output. Figure 14B shows the two smallest voltage steps that were able to evoke the first fast inward current. The one on the left is the control measure (-36mv) and on the right was recorded during brainstem stimulation. Cooling the cervical cord blocked the brainstem evoked hyperpolarization of Vth. The ventral root

recordings from both L2 roots and both L5 roots are shown in each of panels A-C and show that like Figure 1, cooling the cervical partition blocked VR output.

Figure 14C shows when the preparation is washed-out with warm aCSF and the tissue is allowed 10 minutes to return to 25° C. Warming the cervical partition restored brainstem evoked VR output and the accompanying hyperpolarization of Vth to the same degree. Similar results were obtained in all six experiments in which cooling was attempted.

The Motoneurons are Recruited for VR output

In this study three motoneurons were recorded in current clamp mode. This allowed the recording of the depolarization of the cells and action potentials. Figure 15 shows the ventral root recording of a mid-sagittal sectioned spinalcord producing rhythmic alternating activity during brainstem stimulation, and the membrane voltage in the current clamp recording. When electrical stimulation of the brainstem begins, there is an ipsi/ipsi alternating output between rL2 and rL5. This motoneuron located in rL5 fires action potentials synchronized with its associated ventral root activity (grey boxes). Simultaneous ventral root and current clamp recordings demonstrate that the motoneurons that are recorded can be recruited in the VR output (n= 3). Vth hyperpolarization was not analyzed in this recording protocol due to technical constraints outlined in the Methods.

Electrical Stimulation of the Dorsal Root can Induce a Change of Vth

If electrical stimulation of the brainstem could produce a hyperpolarization of Vth without ventral root output, could other types of stimulation also produce a hyperpolarization of Vth of the first fast inward current? For example, electrical

stimulation of the dorsal roots can produce locomotor-like output (Marchetti et al. 2001). In the present study, dorsal root stimulation failed to evoke locomotor-like activity (n= 6). However in 8/8 cells examined dorsal root stimulation (100- 600 μA, 5ms, 1-3 Hz) could produce a Vth hyperpolarization. Figure 16 shows a summary of the data obtained from these eight neurons. Electrical stimulation of the VMM produced a hyperpolarization of Vth of the first fast inward current (light bars). In these same eight neurons, dorsal root stimulation also caused a hyperpolarization of Vth (dark bars). Figure 16 shows that in all cases there was a hyperpolarization of Vth ranging from -2 to -8 mV. In only one case the amount of Vth change was the same in both stimulation paradigms. The different stimulation paradigms produced different Vth modulation but for the sample as a whole neither paradigm consistently produced a larger change in Vth (paired t-test, $p = 0.442$).

5-HT Causes Vth Hyperpolarization Similar To Electrical Stimulation

Fedirchuk & Dai (2004) showed that addition of 5-HT to the lumbar spinal cord of 1-5 day old rats resulted in Vth hyperpolarization of motoneurons. In 4 experiments 5-HT was added to the lumbar bath to compare the Vth hyperpolarization produced by electrical stimulation of the brainstem and 5-HT. Figure 17 shows a summary of the Vth data for four neurons that had a change in Vth, in three neurons the Vth hyperpolarizes and one neuron Vth depolarizes when the brainstem was electrically stimulated. Once the Vth had returned to control, 5-HT was added to the bath (10 – 40 μM at 10μm increments) and five minutes later Vth was measured. 5-HT resulted in changes similar to those produced by electrical stimulation of the VMM. In three neurons the Vth hyperpolarized with both 5-HT addition or VMM stimulation and in one neuron Vth

depolarized when 5-HT was added to the lumbar bath or the VMM was stimulated. The effects of 5-HT were identical to electrical stimulation of the brainstem. 5-HT administration resulted in rhythmic ventral root activity in 2/4 experiments. The effect of 5-HT on Vth could be washed out and Vth recovered to control within minutes as demonstrated in Fedirchuk & Dai (2004).

Addition of 5-HT_{2A} Receptor Antagonist Blocks Vth Hyperpolarization

Fyda & Jordan (1997) have shown that 25 μM ketanserin (5-HT_{2A} receptor antagonist) blocks electrical brainstem locomotor output. It is a reasonable hypothesis that 5-HT is acting through 5-HT_{2A} receptors to produce the brainstem-evoked Vth hyperpolarization described in this study.

Figure 18 demonstrates the ability of ketanserin to block Vth hyperpolarization evoked by electrical brainstem stimulation (n=11). As in previous figures the change in Vth induced by brainstem stimulation is depicted by bars. The open bars represent the change in Vth produced by electrical stimulation of the brainstem prior to application of ketanserin to the thoracolumbar cord partition. The dark filled bars indicate the change in Vth recorded with ketanserin added to the thoracolumbar bath. Ketanserin at 2 μM was unable to block the brainstem evoked Vth hyperpolarization of the neurons but increasing the concentrations to 4- 5 μM was partially able to block the Vth changes. Concentrations ≥ 8 μM were always able to block the Vth hyperpolarization associated with electrical stimulation of the VVM. The effective concentration is consistent with Fyda & Jordan's (1997) findings in that concentrations of ketanserin (25 μM) could block electrically-evoked locomotor activity. In the present study ketanserin blocked ventral root activity when present (n=2).

Recovery of Brainstem Evoked Vth Hyperpolarization

Krawitz et al. (2001) reported there was recovery of Vth to within 1mV of control value within 145 seconds following MLR evoked fictive locomotion in the cat. In the present experiment in 61 cells recovery was monitored, 45 recovered to control. Recovery was monitored until the cell died. Of the 45 that recovered 26 recovered in < 0-1 minute, 9 recovered in 1-2 minutes, 3 recovered in 2-3 minutes, 5 recovered in 3-5 minutes, and 3 recovered in 5-10 minutes. 16 did not recover to control Vth values even in some cases up to 20 minutes later. These neurons were not excluded from the sample. The remaining neurons deteriorated before Vth could return to baseline.

Repetitive Firing of Motoneurons

In some cases motoneurons exhibited multiple fast inward currents on the depolarizing steps. In 11 neurons the fast inward currents were activated more than once during the depolarized portion of the trace. Since the Vth for first fast inward currents was hyperpolarized during electrical stimulation of the brainstem it was expected that the Vth for multiple fast inward currents would hyperpolarize during stimulation as well. Eleven neurons displayed multiple fast inward currents in this study. It was determined that electrical stimulation of the brainstem caused the Vth of second fast inward currents to hyperpolarize (8 of 11 cases). Figure 19 shows a neuron that had multiple fast inward currents, the first fast inward current occurred at –36 mV and the second fast inward current occurring at –26mV during control. During electrical stimulation of the brainstem Vth hyperpolarization occurred with the first fast inward current occurring at –38mV and the second fast inward current occurring at –30 mV. The Vth for the

activation of multiple inward currents hyperpolarized during brainstem stimulation indicates a facilitation of repetitive firing.

Discussion

This study has shown that electrical stimulation of the VMM activates endogenous systems that induce a hyperpolarization of Vth in motoneurons and unidentified ventral horn neurons in the lumbar segments of the neonatal rat. The present study builds upon the state-dependant phenomenon described by Krawitz et al. (2001) in the cat. The present study shows that the phenomenon of Vth hyperpolarization is a mechanism conserved across species and that endogenous systems are able to modulate Vth in the neonatal animal.

Vth Hyperpolarization is Produced by Endogenous Pharmacological Mechanisms

It is important to note that in this study, the change in Vth is produced from endogenous mechanisms activated by electrical stimulation. Cooling the cervical spinal cord blocked the brainstem evoked ventral root output and the Vth change in spinal neurons, indicating that these effects were mediated by descending fibers and not direct activation of spinal neurons by current spread. The use of electrical brainstem stimulation recruited endogenous systems capable of evoking locomotion and Vth hyperpolarization.

Comparison to Vth Hyperpolarization Observed in the Decerebrate Cat

It has been observed that motoneurons and neurons undergo Vth hyperpolarization during electrical stimulation of the VMM that produced a variety of ventral root motor-outputs (ipsi/ipsi alternation, ipsi/contra alternation, rhythmic bursting, tonic activity, and no activity). In this experimental protocol each neuron served as its own control, Vth hyperpolarization occurred with the onset of electrical stimulation of the VMM, and recovered following the end of stimulation. The *in vitro* neonatal rat was

capable of producing a variety of patterns of VR output and Vth hyperpolarization was evident during each pattern observed, and even in the absence of VR output. This study presents the idea that Vth modulation may not be wholly dependant on full locomotion.

The present study used electrical stimulation of the dorsal roots as well as electrical stimulation of the brainstem. It was observed that electrical stimulation of the dorsal root could produce a Vth hyperpolarization without producing a locomotor-like output. Smith et al. (1988), Marchetti et al. (2001) reported that electrical stimulation of the dorsal roots could produce locomotor-like output. Marchettie et al. (2001) hypothesize that since dorsal root stimulation activated the CPG for locomotion in the rat isolated spinal cord, sensory inputs from the periphery can reach the spinal locomotor network and trigger its operation. Since dorsal root stimulation did not produce a locomotor-like output but did produce a Vth hyperpolarization is more evidence that incomplete activation of the locomotor CPG is sufficient for Vth modulation.

Possible Pharmacological Mechanisms Involved

Krawitz et al. (2001) reported that with the onset of locomotion, Vth hyperpolarized and that when locomotion stopped Vth recovered within seconds is consistent with a neuromodulatory substance. Fedirchuk (2001) found that addition of 5-HT (10-12 μM), NA (10-12 μM), and Ach (100μM) hyperpolarized Vth in lumbar motoneurons (Fedirchuk 2001). These experiments involved direct application of 5-HT, NA, and Ach to the bath and showed that activation of receptor systems is enough to generate Vth hyperpolarization, without activation of the CPG.

5-HT and NMDA receptor actions may be critical for the production of rhythmic motor behavior in the mammalian spinal cord MacLean et al. (1998). MacLean et al.

(1998) used 5-HT receptor antagonists to abolish pharmacologically induced rhythmic hindlimb activity. Fyda & Jordan (1997) were able to block electrically-evoked brainstem stimulation using 5-HT_{2A} and 5-HT_7 receptor antagonists, ketanserin and clozapine respectively. Since these receptor antagonists were effective in blocking electrically-evoked locomotor-like activity Fyda & Jordan concluded that 5-HT_{2A} and 5-HT_7 receptors are an integral part of the locomotor network. In the present study only ketanserin (5-HT_{2A}) was examined. Ketanserin was able to block ventral root locomotor-like output (30 μM, n=2) and was able to completely block Vth hyperpolarization in seven cases to partially block in five cases, and was unable to block Vth hyperpolarization in four cases (Figure 18). Concentrations of >6 μM ketanserin were able to completely block the Vth hyperpolarization while concentrations <6 μM ketanserin were partially blocked or failed to block Vth hyperpolarization. If the phenomenon of Vth hyperpolarization is a mechanism used by the CPG to induce locomotor-like activity it seems that blocking the actions of 5-HT will block locomotion and Vth hyperpolarization concurrently. Interpretation of these results also needs to take into consideration the degree of receptor selectivity of the antagonist as outlined in the methods. Work by Fedirchuk (2001) indicates that NA and Ach are also capable of hyperpolarizing Vth. It may be that Na and Ach are capable of directing activating receptor systems capable of producing Vth hyperpolarization. However the role of transmitter systems other than 5-HT in mediating the Vth hyperpolarization have not been explored in the present study.

Comparison to Other Models for Vth

This study reports a development of a preparation for the study of Vth hyperpolarization, however it is not the first to report the modulation of neuronal Vth. There are other reports of Vth hyperpolarization in the literature. Hochman & McCrea (1994) reported that mean motoneuron threshold potential is depolarized in chronic spinal cats compared to spinal intact cats. In a classical conditioning paradigm to decrease the amplitude of the H-reflex in monkeys the mean Vth of spinal motoneurons became depolarized (Carp & Wolpaw 1994). Cleary et al. (1998), have shown that the Vth of motoneurons in aplysia was hyperpolarized the day following a long-term sensitization of the siphon reflex. Beaumont & Gardiner (2003) have reported that spike threshold was more hyperpolarized in slow motoneurons of adult rats following a 16-week treadmill-training program. Martin (2002) examined the electrophysiological properties of lamprey motoneurons during fictive swimming. Interestingly it was found that though many different membrane properties change during fictive lamprey swimming like rheobase, the (F-I) relationship and amplitude of the AHP, spike threshold did not change.

Possible Conductances Underlying Vth Hyperpolarization

Recently, Dai et al. 2002 has shown that modulation of g_{Na} and $g_{K(DR)}$ (in a simulated cell) can hyperpolarize Vth by increasing the inward current at subthreshold potentials. There were four conductance changes that could hyperpolarize Vth with only small changes in spike height (<8mV) and width (<1ms). The four conductance changes were (1) increasing the initial segment maximum conductance of g_{Na} by 50%, (2) shifting the initial segmental g_{Na} voltage dependency in the hyperpolarizing direction by 3mV, (3) reducing the maximum conductance of initial segment $g_{K(DR)}$ by 70%; and (4) shifting

the initial segment $g_{K\,(DR)}$ voltage dependency in the depolarizing direction by 5 mV. These conductances were found to be most effective in the initial segment.

Modulation of the other major conductance's; g_{KAHP}, $g_{Na\ persistent}$, g_{Kdr}, g_{KcaK}, g_{KIR}, g_{KA}, g_{H}, g_{CAt}, g_{CaN}, g_{CaL} either had relatively less or no effect on Vth or changed the spike height (>8mV) or width (>1ms). Dai et al. (2002) suggest that, "the selective modulation of Na^+ and/or delayed-rectifier channels in the initial segment are candidate ionic mechanisms for the hyperpolarization of the Vth that occurs in adult cat spinal motoneurons during fictive locomotion." Therefore it is hypothesized that hyperpolarization of Vth occurs by the modulation of fast Na^+ channels.

In addition to modulation of the fast sodium current underlying action potentials, it is also possible that other conductances could be involved in the monoamine induced Vth hyperpolarization. Reducing a potassium conductance could also hyperpolarize Vth, although to a lesser degree than direct manipulation of sodium channels (Dai et al. 2002).

Phosphorylation of Na^+ Channels Produces a Decreased Inward Current

Phosphorylation and dephosphorylation of tyrosine residues on Na^+ channels modulate Na^+ channel inactivation. Phosphorylation of Na^+ channels by direct activation of PKA and PKC has been observed (Cantrell & Catterall 2001). Phosphorylation by PKC results in reduced Na^+ current and slowed inactivation. Similarly phosphorylation by PKA resulted in a 25 – 40 % reduction of Na^+ channel activity, (Cantrell & Catterall 2001). PKA phosphorylation by PKA shifts Na^+ channels to a null gating mode in which depolarization has a low probability of transition to the open state (Cantrell & Catterall 2001). The second messenger system of G proteins can regulate the voltage-dependant gating and persistent Na^+ current of Na^+ channels (Cantrell & Catterall 2001).

Recent work by Carr et al. (2002) has shown that in brain pyramidal neurons 5-$HT_{2A/C}$ receptor activation reduced Na^+ currents by reducing maximal current amplitude and shifting inactivation voltage dependence. The effects were thought to occur by Gq activation of phospholipase C, and activation of PKC. While Carr et al. (2002) did find current modulation by 5-HT that they did not find a change in Vth. This does not necessarily refute the hypothesis that 5-$HT_{2A/C}$ receptor activation will hyperpolarize Vth of spinal neurons.

Preliminary experiments by Fedirchuk & Dai (2004) exploring the activation of PKC pathways in motoneurons resulted in the hypothesis that phosphorylation of Na^+ channels via PKC will result in Vth hyperpolarization. Initial experiments show that application of OAG (a PKC activator) results in Vth depolarization. The use of a PKC inhibitor to examine if it can block the serotonergic activation of Vth hyperpolarization via PKC. Preliminary data has shown there is no effect of the PKC inhibitor on Vth hyperpolarization.

Limitations of Study

This thesis describes the development of a new preparation for the study of Vth modulation. One of the limitations of this study is that the depolarizing step used to estimate Vth was set at 2 mV. This allowed any changes in Vth < 2 mV to go undetected. Future studies must include holding potential steps of smaller magnitude along with direct measurement of Vth in current clamp.

Another concern raised in this study was the large amount of current used to evoke locomotor-like activity due to volume conduction into lumbar regions and directly activating lumbar circuitry for locomotor-like activity. It was found that with proper

grounding current spread could be avoided. For a detailed discussion of these issues see Zaporozhets et al. (2004). When improper grounding techniques were used it was found that the intracellular holding potential leak current would fluctuate rapidly. When proper grounding techniques were used (e.g. chloride grounds, grounding between electrode and lumbar compartment) then the intracellular holding potential leak current would remain steady during brainstem stimulation periods. It is believed that this preparation required high current strength to evoke locomotor-like activity and Vth hyperpolarization because a large portion of the brainstem need to be activated. Fyda & Jordan (1997) have shown that electrical stimulation of the brainstem results in NA, DA and 5-HT to be released into the ventral horn of the thoracic – lumbar spinal cord. Zaporozhets et al. (2003) have also shown that blockade of dopaminergic, serotinergic, or adrenergic receptors can abolish brainstem evoked locomotion. It must be taken into consideration that the high current needed to evoke locomotion activates many systems within the spinal cord and the pharmachological mechanism of Vth hyperpolarization remains unclear.

The finding of Vth hyperpolarization reported by Krawitz et al. (2001) was reported using current clamp recordings. Current clamp recordings were not used in this study due to limitations of the Axo-patch 1-D to accurately record action potentials. Current clamp recordings must be utilized to ensure proper comparisons of the Vth hyperpolarization in the neonatal rat and cat models.

Further Studies

Further studies examining Vth hyperpolarization are needed to explore examination of PKC and PKA pathways, examination of Vth variability among cells, and exploration of the effects of NA and Ach on Vth hyperpolarization. Future studies

should include the use of additional 5-HT receptor antagonists and agonists in an effort to elucidate mechanisms, and the exploration of other transmitter systems able to modulate Vth.

Recent work by Zaporozhets et al. (2004) has shown that reliable electrical stimulation of the brainstem can evoke locomotor-like motor output using a very low (1-10) kOhm electrode, with stimulation parameters of 1-3 mA, of 5-15 ms and 1-3 Hz. Unfortunately this equipment was not available during the experimental procedure of this thesis. This thesis only employed a 5ms stimulation and used much higher resistance (10-150 kOhm) electrodes, therefore it is believed that locomotor-like activity only occurred in optimal conditions. Future studies would include the above protocols from Zaporozets et al. (2004) to examine locomotor-like motor output and associated Vth hyperpolarization.

Conclusion

Krawitz et al. (2001) recent discovery of a state-dependant change in Vth introduced a new mechanism in modulating neuronal excitability. This thesis has determined that Vth hyperpolarization is a feature of many motor outputs and is not wholly dependant on a locomotor-like VR output. It has been shown that electrical stimulation of the neonatal rat brainstem and dorsal roots can produce a Vth hyperpolarization independent of motor output. It is not known at this time but we speculate that Vth hyperpolarization is modulated through the action of 5-HT_{2A} receptors on motoneurons. Vth hyperpolarization occurs in the cat and in the neonatal rat and is therefore a mechanism that is conserved across species. Vth hyperpolarization occurred

in many different motor outputs and it is hypothesized that this is a mechanism that is used as a way to increase motoneuron excitability pursuant to behaviour.

We are just beginning to understand the multiple mechanisms responsible for the modulation of channel kinetics, currents and voltage sensitivity. The idea that the motor-system can modulate neuronal excitability differently for specific behaviours is emerging. This would have important ramifications as neuron input-output relations might be modulated quickly and effectively and the recruitment of a single neuron during different specific behaviours is possible.

Figure legends

Figure 1: A. The half-center model as proposed by Graham Brown (adapted from Lundberg 1981). Solid circles represent inhibitory synapses and open triangles excitatory synapses. B. The reciprocal organization between flexors and extensors (adapted from Gossard & Hultborn 1991). A single interneuron diagrammed represents a chain of interneurons. C. The Miller & Scott model illustrating the electrical connections between cells (adapted from Miller & Scott 1977). RC indicates a Renshaw cell, IaIN indicates a Ia inhibitory interneuron and MN indicates a motoneuron. D. The Pearson & Duysens model for the swing generator (adapted from Pearson & Duysens 1976).

Figure 2: A. The Kling & Szekely ring model. Left side: ring model of three neurons. Right side: inhibitory trough of one neuron (eg, C) coincides with spike train (F) of another neuron (eg. A), which will disinhibit neuron B. Duration of recovery time (R) is influenced by excitability of neurons. P=period length (adapted from Grillner 1981).

Figure 3: Diagram of the brainstem-spinal cord preparation. The stimulating electrode was placed on the ventral medulla. The cervical spinal segments were partitioned from the brainstem and lower spinal segments. Lumbar-ventral roots are shown where suction electrodes were attached. Also shown are the motoneuron and the intracellular recording electrode. Identification of motoneurons was done by switching a recording ventral root suction electrode to a stimulating electrode. Antidromic activation could be seen as a

short latency all-or-none fast inward current following the stimulus and the stimulus intensity recorded.

Figure 4: Panel A shows locomotor-like ventral root activity produced by electrical stimulation of brainstem (VMM, 1mA, 1ms, 1Hz for 140 seconds). When the cervical cord was cooled to 4°C (B), locomotor-like output could not be evoked (same stimulation parameters as A). When the cervical cord was rewarmed (C) locomotor-like output returns.

Figure 5: Electrical stimulation of the brainstem can induce a locomotor-like ventral root activity and hyperpolarization of Vth. Panel A shows the ventral root records (VR) prior to, and during electrical stimulation of the ventromedial medulla (indicated by bar). The brainstem stimulation rapidly induced rhythmic activity that exhibited alternationbetween right and left sides, and between ipsilateral L2 and L5 VRs. This pattern of VR activity was considered consistent with locomotor-like activity. An antidromically identified rL2 motoneuron was recorded throughout the period shown in A. Panel B shows ht determination of the threshold of activation (Vth) of the motoneuron. In voltage clamp, the cell was held at an initial holding potential of –60 mV, a steps to more depolarized holding potentials were successively applied. Prior to brainstem stimulation, (B, left traces), a depolarizing step to –32 mV was insufficient to evoke a fast inward current, while the next stepto –34 mV activated a large, fast inward current in the membrane current (Im) record. Therefore, Vth was considered to be –34 mV. Only Vth step is illustrated. During the brainstem stimulation depicted in A, Vth was re-assessed and a

smaller depolarizing step (to –38mV), was sufficient to activate the fast inward current. This change represents a –4mV hyperpolarization of Vth during the brainstem-evoked locomotor-like VR activity.

Figure 6: The upper plot (**A**) shows the absolute Vth prior to brainstem stimulation (left points), and during the locomotor-like activity (right points). Measurements from the same neurons are connected by a line. Antidromically indentified lumbar motoneurons have open symbols and dashed lines, while unidentified lumbar ventral horn neurons have solid symbols and lines. The data from these cells is re-plotted in (**B**) as the relative change in Vth during the locomotor-like activity and sorted based on the amplitude of the change (lower plot). One neuron (labeled 1) showed no change in Vth, and 10/11 showed a hyperplorization of Vth (-2 to –18 mV).

Figure 7: Vth hyperpolarization during ipsi/contra activity (n=9). Figure A displays a summary of Vth data for 9 cells during control and electrical stimulation of the VMM that produced ipsi/contra motor output. Note the general trend of Vth hyperpolarization during stimulation. Figure B displays the same data re-plotted as the amount of relative Vth hyperpolarization. Cell1 had a Vth hyperpolarization of -4mV and Cell 9 had a Vth hyperpolarization of -18 mV.

Figure 8: Vth hyperpolarization during ipsi alternating output (n= 6). Note the general trend of Vth hyperpolarization. Figure A displays a summary of Vth data for 6 cells during control and during electrical stimulation of the VMM that produced and ipsi

alternating output. Figure B displays the same data re-plotted displaying the relative Vth for each cell. Cell1 had a Vth hyperpolarization of 0 and cell 6 had a Vth hyperpolarization of -8mV.

Figure 9: Vth hyperpolarization during rhythmic motor output. (n=13). Figure A displays a summary of Vth data for 13 cells during control and during electrical stimulation of the VMM that produced a rhythmic output. Figure B displays the same data re-plotted showing the relative Vth hyperpolarization. For cell one Vth depolarized +2mV and in cell two the Vth had 0 Vth change. Cell three Vth hyperpolarizes –2mV. Note the general trend of Vth hyperpolarization in most cells.

Figure 10: Vth hyperpolarization during tonic activity (n=8). Figure A displays a summary of Vth data for 13 cells during control and during electrical stimulation of the VMM that produced a tonic output. Figure B displays the same data re-plotted showing the relative Vth hyperpolarization. For cell one Vth did not change and in cell 8 Vth hyperpolarized -6mV. Note the general trend of Vth hyperpolarization.

Figure 11: Vth hyperpolarization during no ventral root activity (n=46). Figure A displays a summary of Vth data for 46 cells during control and during electrical stimulation of the VMM that produced no ventral root output. Figure B displays the same data re-plotted showing the relative Vth hyperpolarization. For cell one the Vth depolarized 2mV and in cell 46 the Vth hyperpolarized -16 mV. Note the general trend of Vth hyperpolarization.

Figure 12: Summary of the relative Vth changes for 16 cells in which brainstem stimulation produced VR output that changed during the recording period. Repeated stimulation with the same protocols produced different motor output and Vth hyperpolarization.

Figure 13: Example of ventral root output and the amount of Vth hyperpolarization versus the amount of electrical stimulation of the VMM. The amount of current increases during each period (100-500 μA). The amount of Vth hyperpolarization increased with increasing current. The ventral root activity is not present until 400 μA (rL2) and a rhythmic motor output is produced. Increasing the stimulation to 500 μA resulted in ipsi/contra output of rL2 and lL2. Increasing stimulation did not produce increased Vth hyperpolarization.

Figure 14: Panel A displays a -2 mV Vth hyperpolarization during electrical stimulation (1mA, 5ms, 1Hz) of the VMM and produced a small tonic increase in VR output. In panel B the cervical cord was cooled to 4°C. Note that with the same stimulation parameters as in A there is no change in Vth in the same cell. Notice that the ventral root output is abolished. Panel C shows the same preparation 10 minutes later. The cervical cord has been rewarmed 22°C and the stimulation parameters are the same as in A and B. The Vth hyperpolarization of 2 mV recovers, as does the ventral root activity.

Figure 15: Intracellular current clamp recording of an antidromically identified motoneuron. Concurrent ventral root recordings are shown in the lower traces. Ventral root recordings and the intracellular records are silent until stimulation begins. Grey boxes indicate areas where increased ventral root output coincided with increase motoneuron spiking. Motoneuron spiking is evident with ventral root activity. Subthreshold depolarizing potentials from the CPG are also visible.

Figure 16: Shows the relative amount of Vth hyperpolarization from control in the same cell when electrical stimulation was delivered to the VMM and subsequently to the rL5 dorsal root. Notice that there is a hyperpolarization of Vth with either stimulation. The amount of hyperpolarization was not greater with any single stimulus.

Figure 17: Shows a summary of the Vth of 4 cells during 4 different conditions. First the Vth is recorded during control. Secondly the Vth is recorded during electrical stimulation of the ventromedal medulla. In all but one case Vth hyperpolarizes. The cells were allowed to return to control and Vth was recorded. Thirdly 5-HT (10-40 μM) was added to the bath. This caused a hyperpolarization of Vth in all but one case. In cell one and 2 5-HT caused the same degree of Vth hyperpolarization as electrical stimulation.

Figure 18: A summary of Vth's from 16 cells where ketanserin, a 5-HT_{2A} antagonist, was added to the lumbar bath. The gray bars indicate the concentration of ketanserin (μM) was added to each cell. The black bars indicate the amount of Vth hyperpolarization with electrical stimulation of the VMM before the ketanserin was

added. Note: in all cases Vth hyperpolarized with stimulation. The white bars indicate the amount of Vth hyperpolarization once the ketanserin was added to the bath. Instances where there was no Vth change are labeled 0. Note how ketanserin concentrations greater than 6 μM blocked the Vth hyperpolarization.

Figure 19: Shows voltage clamp recording from an antidromically identified motoneuron during control and during electrical stimulation of the VMM. Figure A is the voltage clamp recording during control, displayed is the first fast inward current at 24 mV depolarization, also displayed is the second fast inward current that occurred with 34 mV of depolarization. Figure B shows the voltage clamp recording from the same motoneuron during stimulation. Notice the first fast inward current Vth hyperpolarized (-2mV) and is evoked with only 22 mV of depolarization. Also notice that the Vth for the second fast inward current also hyperpolarizes (-4mV) vs. (-34mV control). This is an example of the threshold for multiple fast inward currents becoming hyperpolarized during electrical stimulation of the brainstem.

Table 1: On the left find the correct temperature and across the top find the correct pressure. Where the two columns intersect is the amount of O_2 saturation of water. This chart does not correct for salinity. At a temperature of 4°C and a pressure of 1511 mmHg (what the aCSF was pressurized to) this table shows an oxygen saturation of 26.1 mg O_2 per liter of water. Normal 95% O_2 bubbled aCSF is at room temperature and not pressurized, which would result in 8.6 mg O_2 per liter of water.

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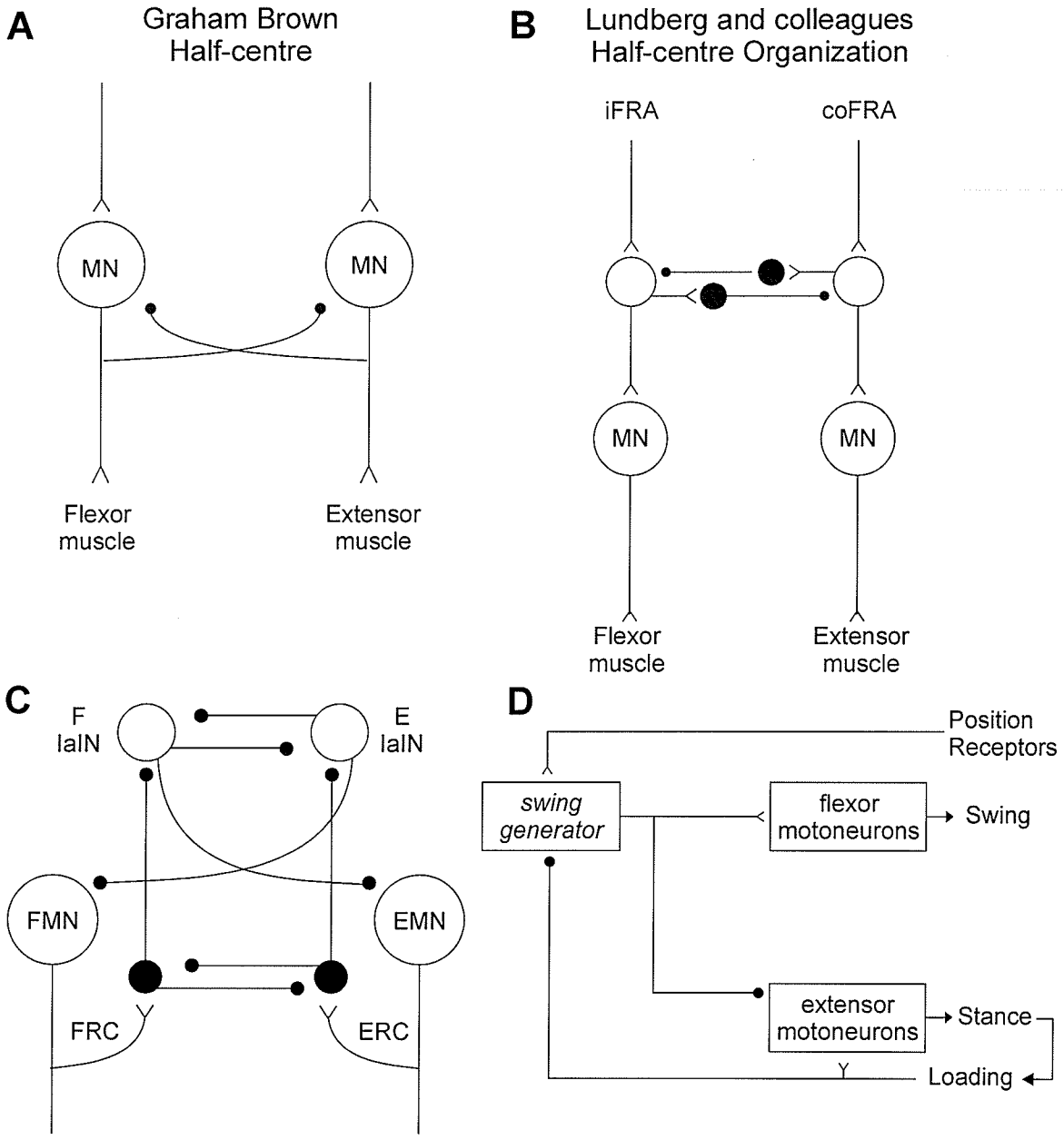

Figure 1
A. The half-center model as proposed by Graham Brown (adapted from Lundberg, 1981). Solid circles represent inhibitory synapses and open triangles excitatory synapses. **B**. The reciprocal organization between flexors and extensors (adapted from Gossard and Hultborn, 1991). A single interneuron diagrammed represents a chain of interneurons. **C.** The Miller and Scott model illustrating the electrical connections between cells (adapted from Miller and Scott, 1977). RC indicates a Renshaw cell, IaIN indicates a Ia inhibitory interneuron and MN indicates a motoneuron. **D**. The Pearson and Duysens model for the swing generator (adapted from Pearson and Duysens, 1976).

A

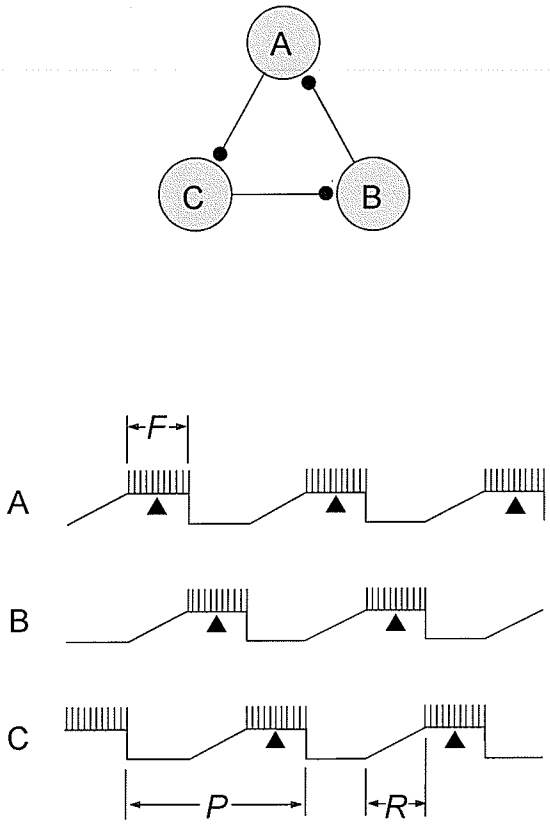

Figure 2

A. The Kling and Szekely ring model. Left side: ring model of 3 neurons. Right side: inhibitory trough of 1 neuron (eg., C) coincides with spike train (F) of another neuron (eg. A), which will disinhibit neuron B. Duration of recovery time (R) is influenced by excitability of neurons. P=period lenght (adapted from Grillner, 1981).

Methods

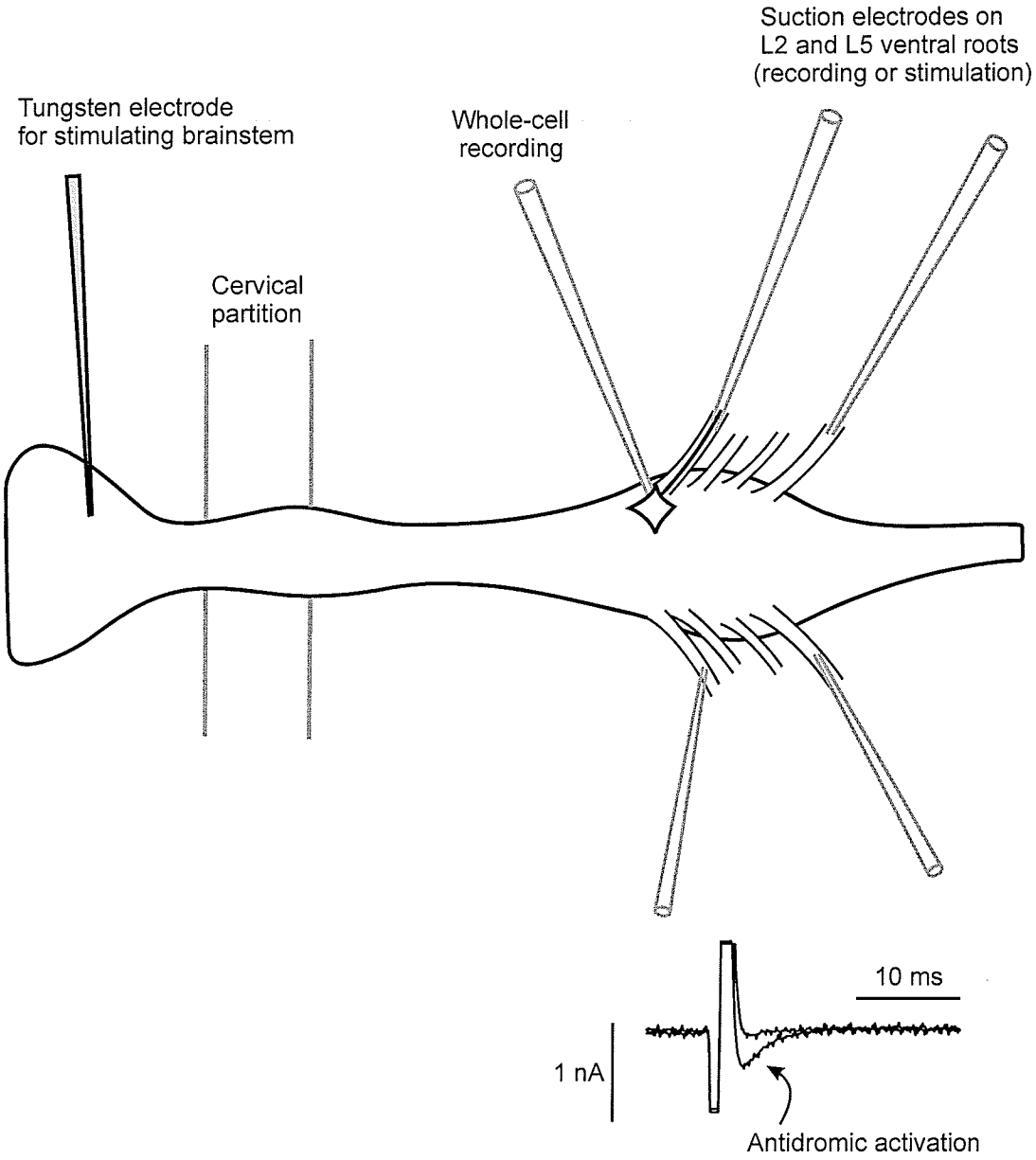

Figure 3
Diagram of the brainstem-spinal cord preparation. A stimulating electrode is placed in the VMM. Suction electrodes are attached on L2 nd L5 VR. Diagramed is a lumbar motoneuron and the attached whole-cell recording electrode. Antidromic activation of motoneurons was acomplished by switching a recording ventral root suction electrode to a stimulating electrode. The short latency all-or-none fast inward current following the stimulus indicated that the cell was a motoneuron.

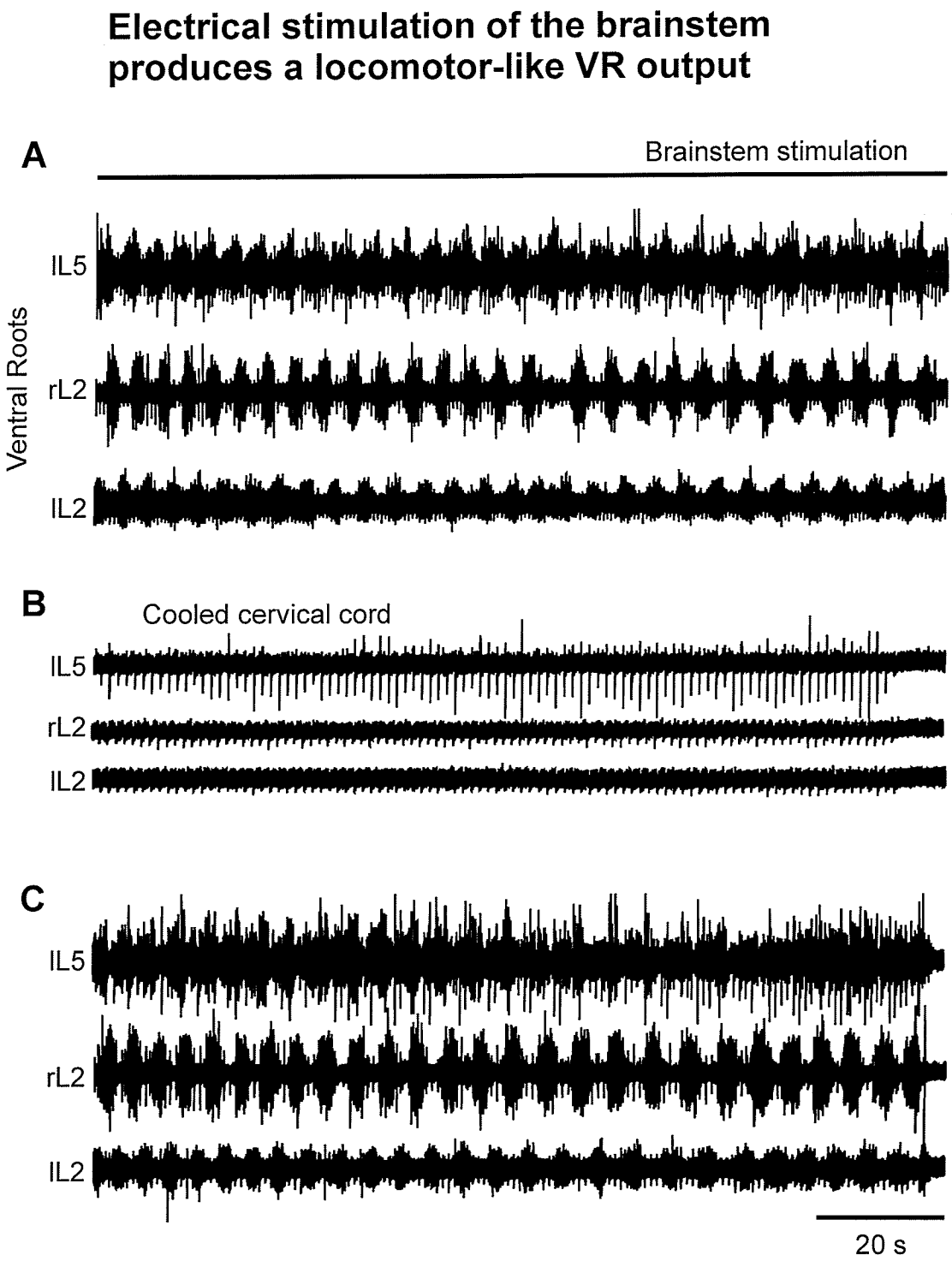

Figure 4
Panel **A** shows locomotor-like ventral root activity produced by electrical stimulation of the brainstem (ventromedial medulla, 1mA, 1ms, 1Hz for 140 seconds). When the cervical cord was cooled to 4°C (**B**), locomotor-like output could not be evoked (same stimulation parameters as A). When the cervical cord was rewarmed [**C**] locomotor-like output returns.

Electrical stimulation of the brainstem evokes a Hyperpolarization of voltage threshold

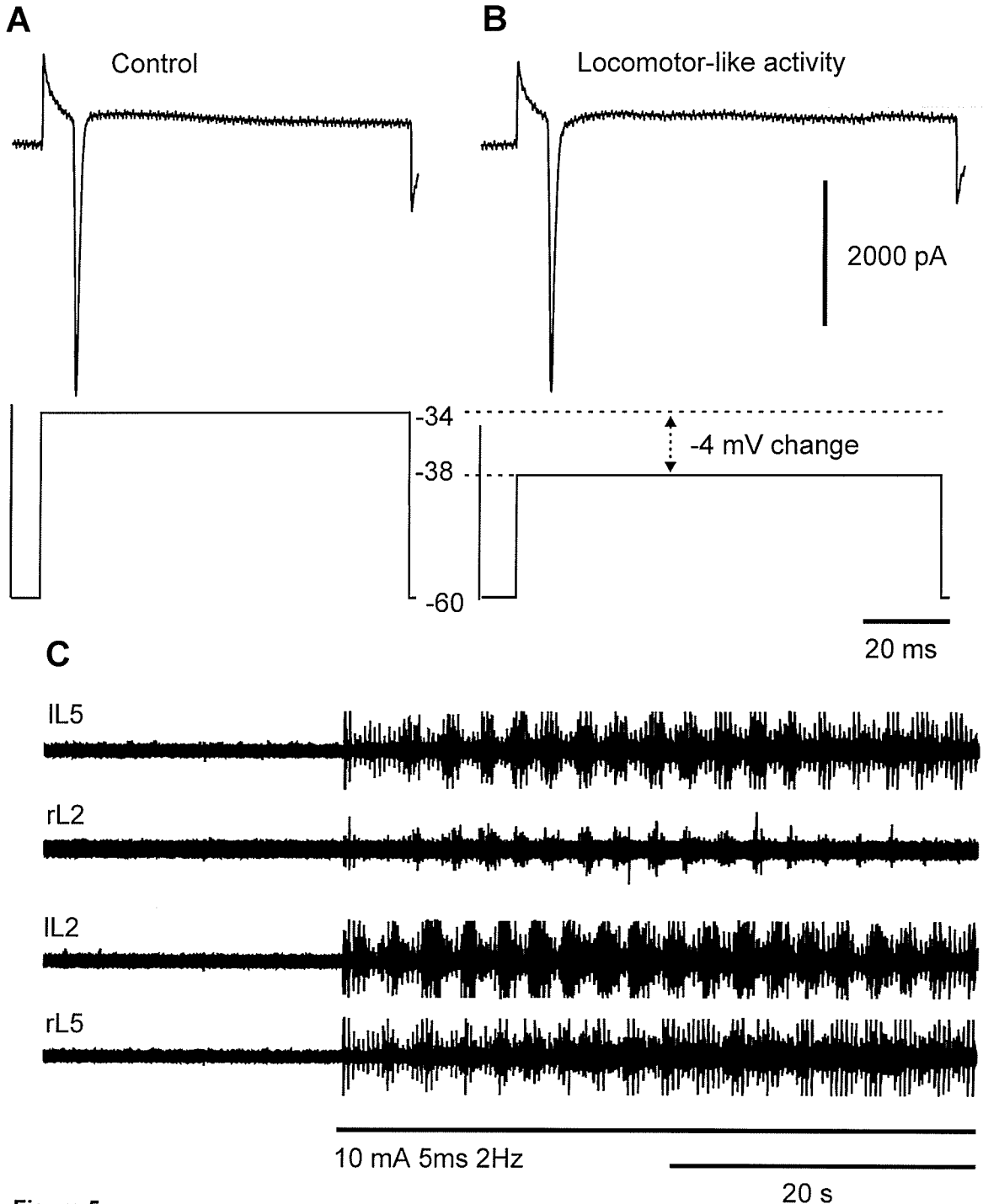

Figure 5
Voltage clamp recordings from an antidromically identified rL2 motoneuron. Vth was estimated by determining the minimum voltage step able to induce a fast inward current. Vth measurements from control periods (**A**) and during electrical stimulation of the brainstem (**B**) were compared. Note sub-threshold and supra-threshold voltage steps are not illustrated in A or B. Panel **C** displays the VR recording.

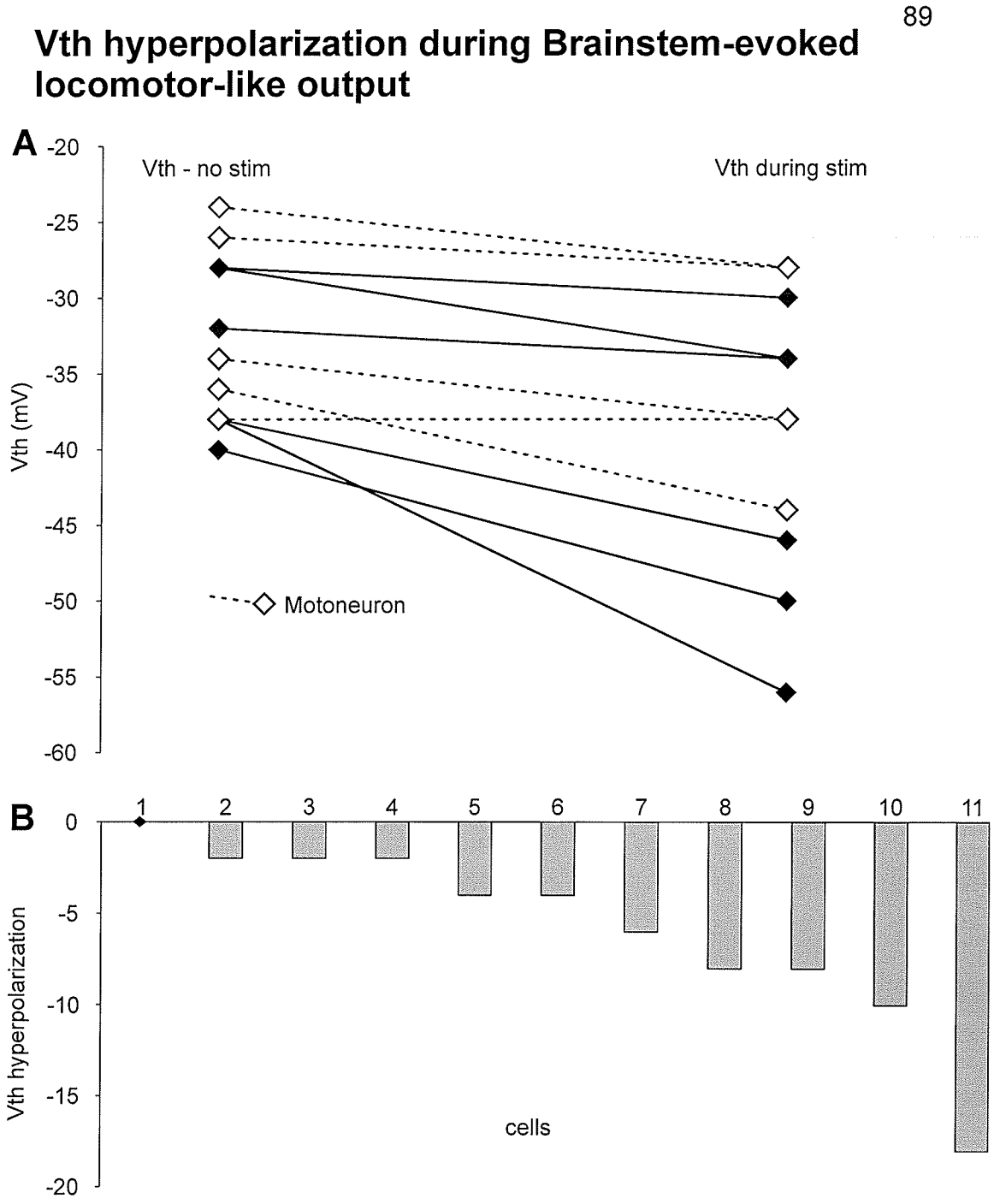

Figure 6
Vth hyperpolarization during electrical stimulation of the VMM that evoked locomotor-like output (n=11). Figure **A** displays a summary of Vth data of 11 cells (identified and unidentified) during control conditions and during stimulation of the ventromedial medulla that produced locomotor-like behavior. Note the general trend of Vth hyperpolarization. Figure **B** displays the same data re-plotted with the relative Vth hyperpolarization during stimulation. Cell 1 had 0 voltage change and cell 11 had a Vth change of -18 mV.

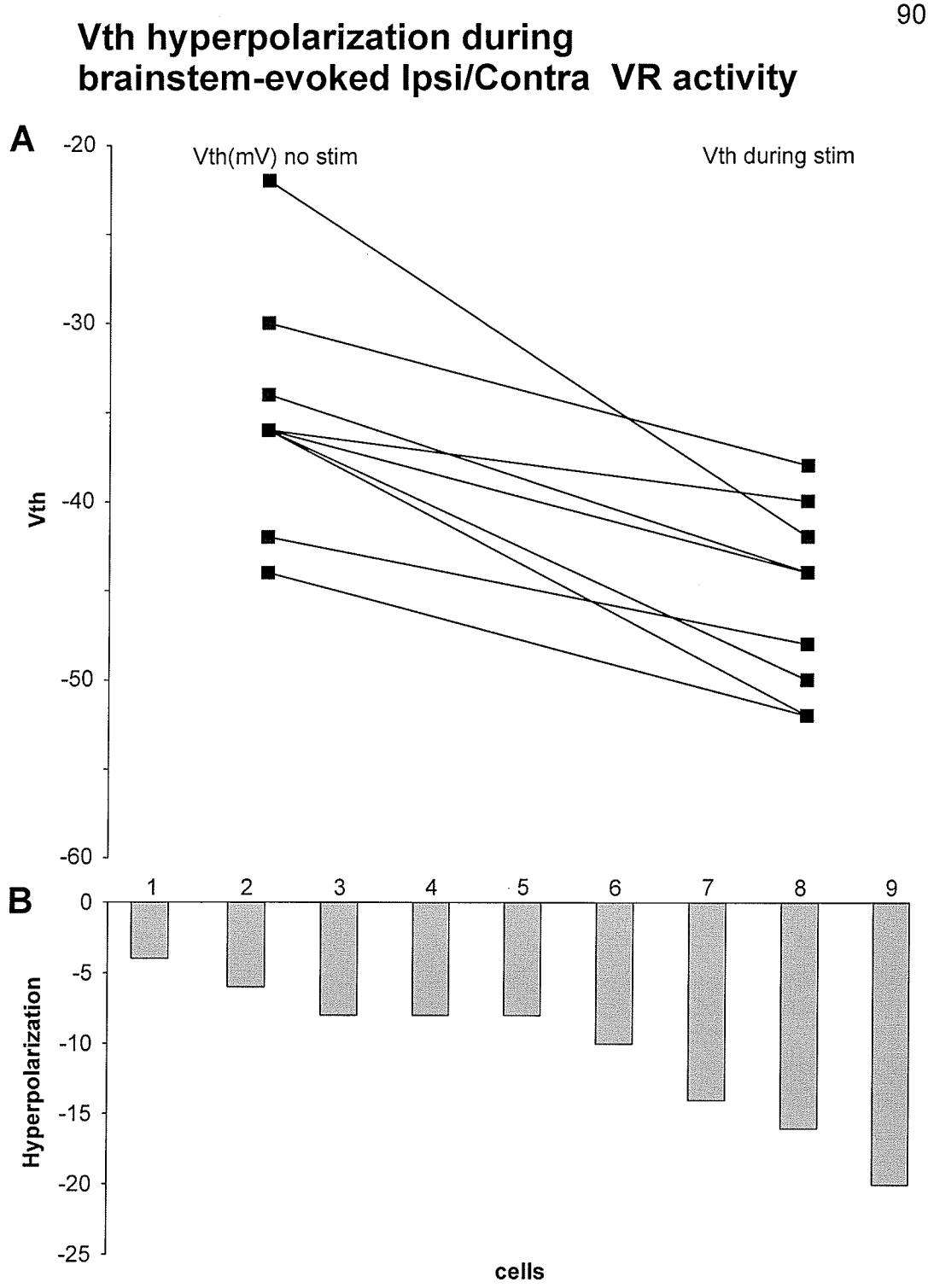

Figure 7
Vth hyperpolarization duringelectrical stimulation of the VMM that evoked ipsi/contra VR activity (n=9). Figure **A** displays a summary of Vth data for 9 cells during control and electrical stimulation of the VMM that produced ipsi/contra motor output. Note the general trend of Vth hyperpolarization during stimulation. Figure **B** displays the same data re-polotted as the amount of relative Vth hyperpolarization. Cell1 had a Vth hyperpolarization of -4mV and Cell 9 have Vth hyperpolarization of -18 mV.

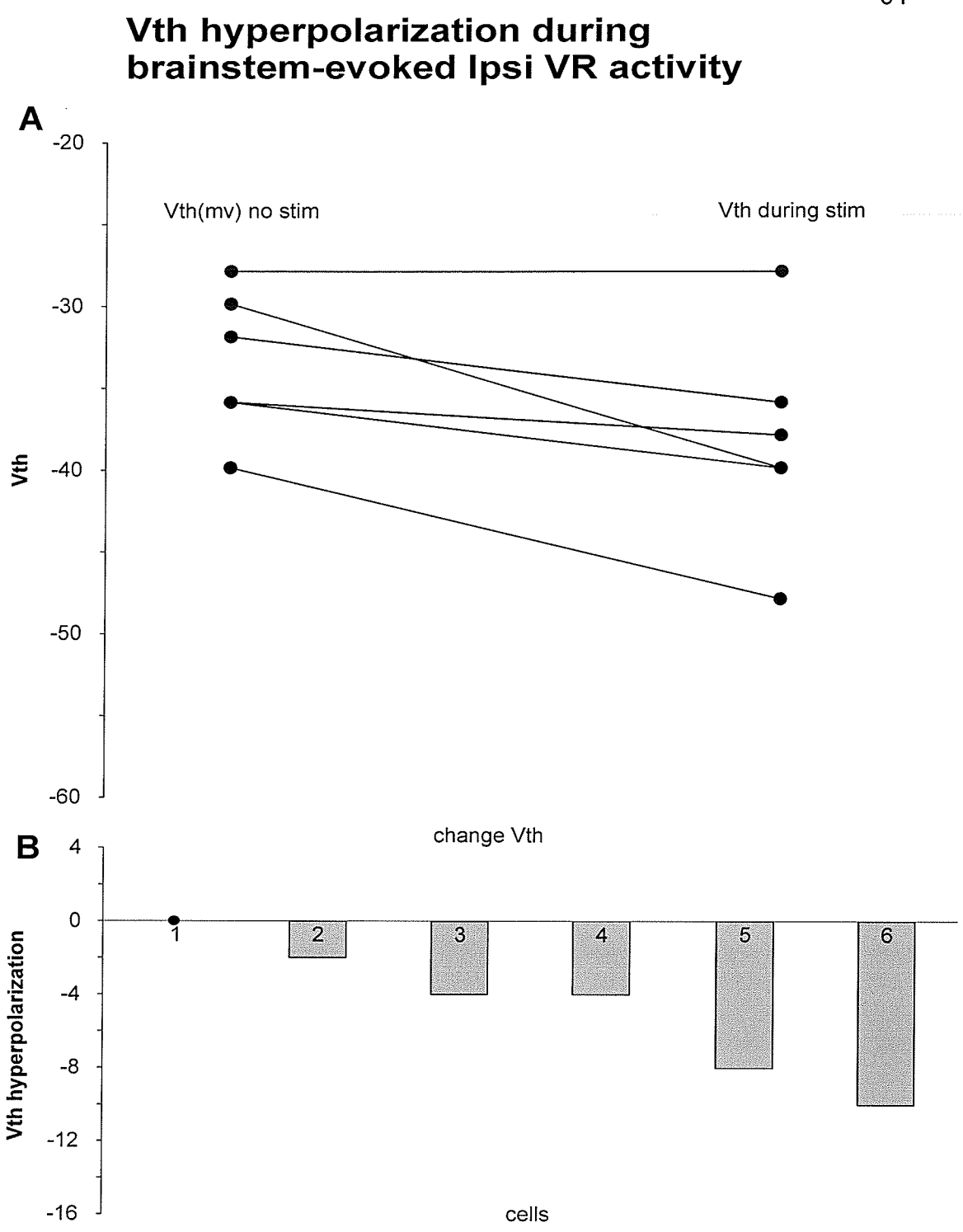

Figure 8
Vth hyperpolarization during ipsi/ipsi behavior (n= 6). Note the genral trend of Vth hyperpolarization. Figure **A** displays a summary of Vth data for 6 cells during control and during electrical stimulation of the ventromedial medulla that produced and ipsi/ipsi output. Figure **B** displays the same data re-plotted displaying the relative Vth for each cell. For neuron 1Vth changed 0 mV and cell 6 had a Vth hyperpolarization of -8 mV.

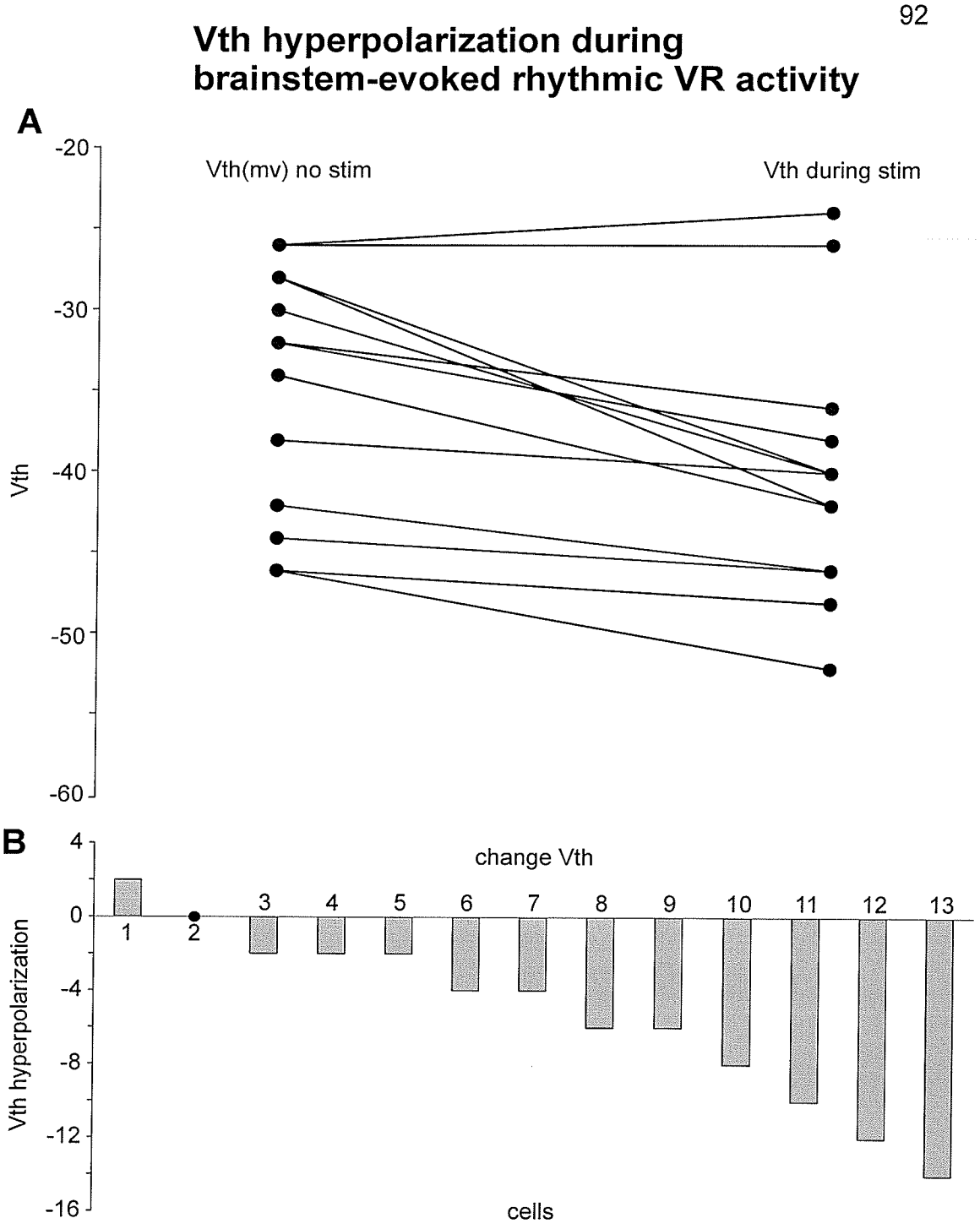

Figure 9
Vth hyperpolarization during rythmic motor output. (N=13). Figure **A** displays a summary of Vth data for 13 cells during control and during electrical stimulation of the ventromedial medulla that produced a rhythmic output. Figure **B** displays the same data re-plotted showing the relative Vth hyperpolarization. For cell 1 the Vth depolarized and in cell 2 the Vth had 0 Vth change. Note the general trend of Vth hyperpolarization.

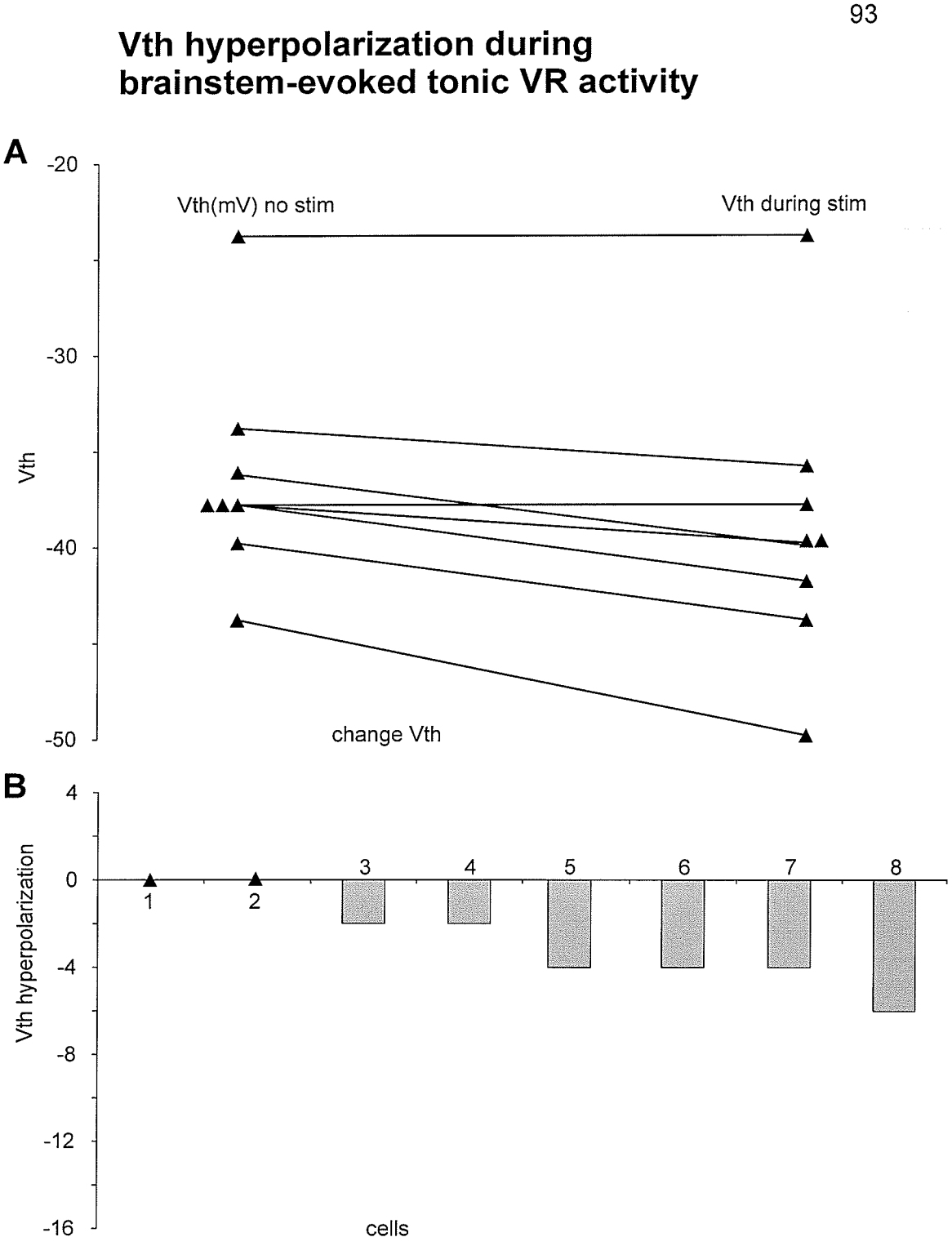

Figure 10
Vth hyperpolarization during tonic activity (n=8).Figure **A** displays a summary of Vth data for 13 cells during control and during electrical stimulation of the ventromedial medulla that produced a tonic VR output. Figure **B** displays the same data re-plotted showing the relative Vth hyperpolarization. For cell 1 the Vth was unchanged 0 mV and in cell 8 the Vth hyperpolarized -6mV. Note the general trend of Vth hyperpolarization.

Vth hyperpolarization in the abscence of VR output

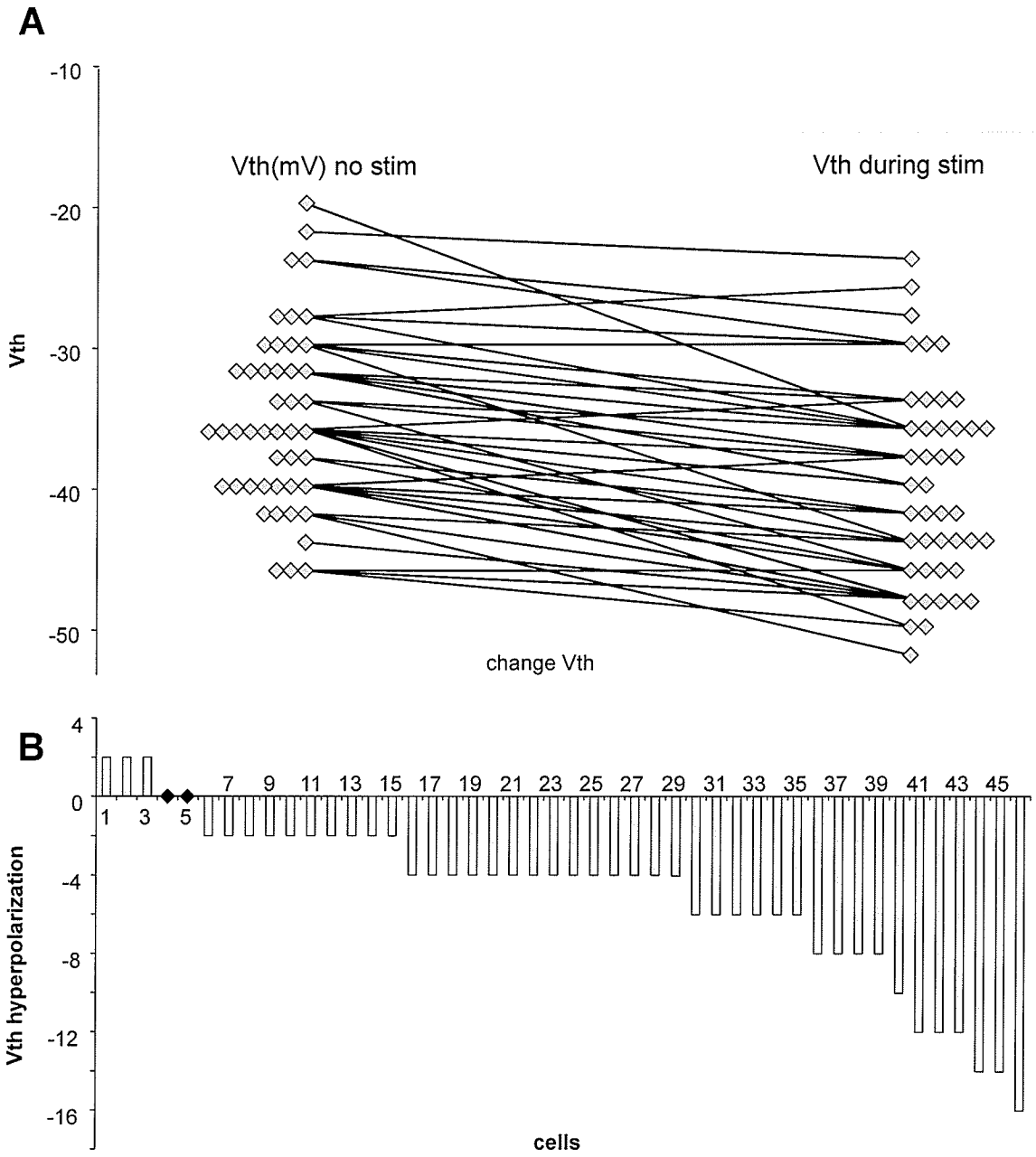

Figure 11
Vth hyperpolarization during no ventral root activity (n=46). Figure **A** displays a summary of Vth data for 46 cells during control and during electrical stimulation of the ventromedial medulla that produced no ventral root output. Figure **B** displays the same data re-plotted showing the relative Vth hyperpolarization. For cell 1 the Vth depolarized 2mV and in cell 46 the Vth hyperpolarized -16 mV. Note the general trend of Vth hyperpolarization.

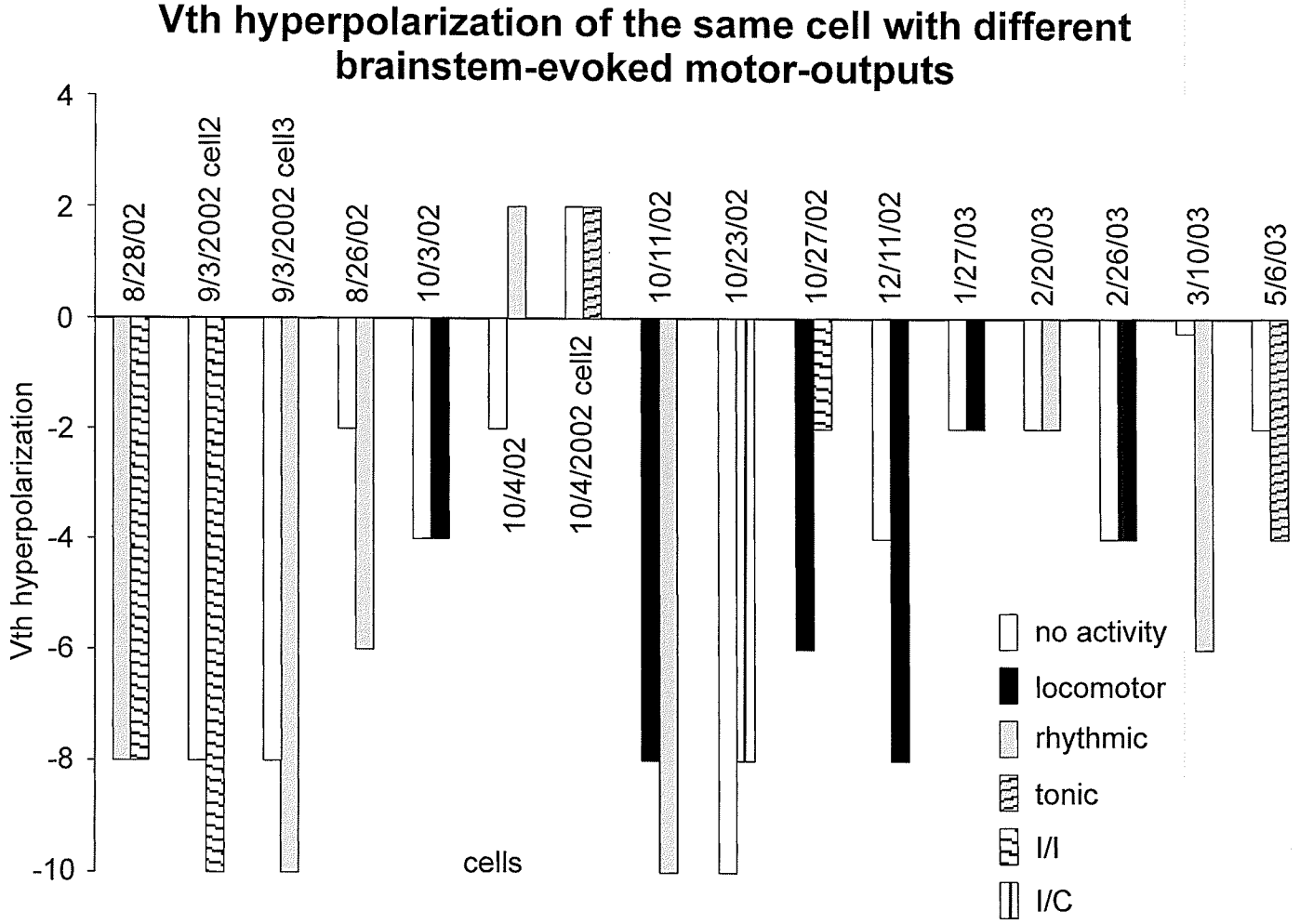

Figure 12
Summary of the relative Vth changes for 16 cells in which brainstem stimulation produced VR output that changed during the recording period. Repeated stimulation with the same protocols produced different motor output. Shown is the relative Vth changes in 16 cells where change in Vth was similar in the same cell regardless of the ventral root behavior.

Hyperpolarization of Vth evoked at stimulus intensities subthreshold for rhythmic motor output.

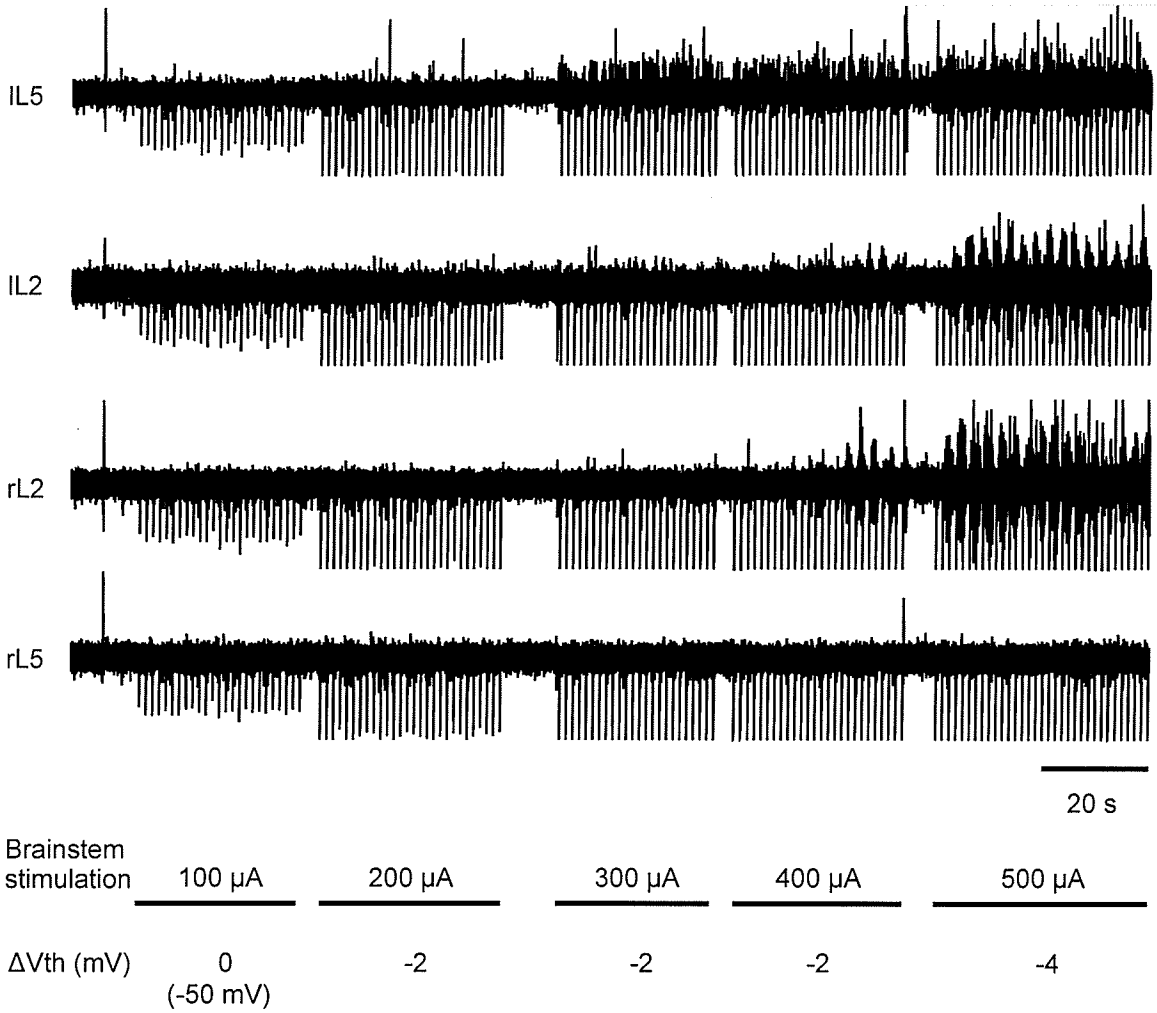

Figure 13
Figure 13 is an example of Vth hyperpolarization induced at brainstem stimulation intensities that were sub-threshold for inducing rhythmic VR activity. Bouts of brainstem stimulation of increasing intensity are indicated by the bars below the ventral root (VR) records. A whole-cell recording of lumbar neuron was simultaneously obtained (not illustrated), and its Vth assed prior to the brainstem stimulations, and during each bout of brainstem stimulation (change indicated below bars). 100 µA stimulation produced shock artifacts in the VR records, but failed to evoke either VR activity or an alternation of Vth. At 200 µA, a -2mV hyperpolarization of the neuron was seen, but VR activity was not evident. Stimulation at 300 or 400 µA did not induce a larger hyperpolarization of Vth, but did evoke tonic, and rhythmic activity respectively. Stimulation at 500 µA, rapidly induced ipsi/contra alternating activity and a -4 mv hyperpolarization of Vth.

Cooling the cervical spinal cord blocks the brainstem stimulation evoked change in Vth

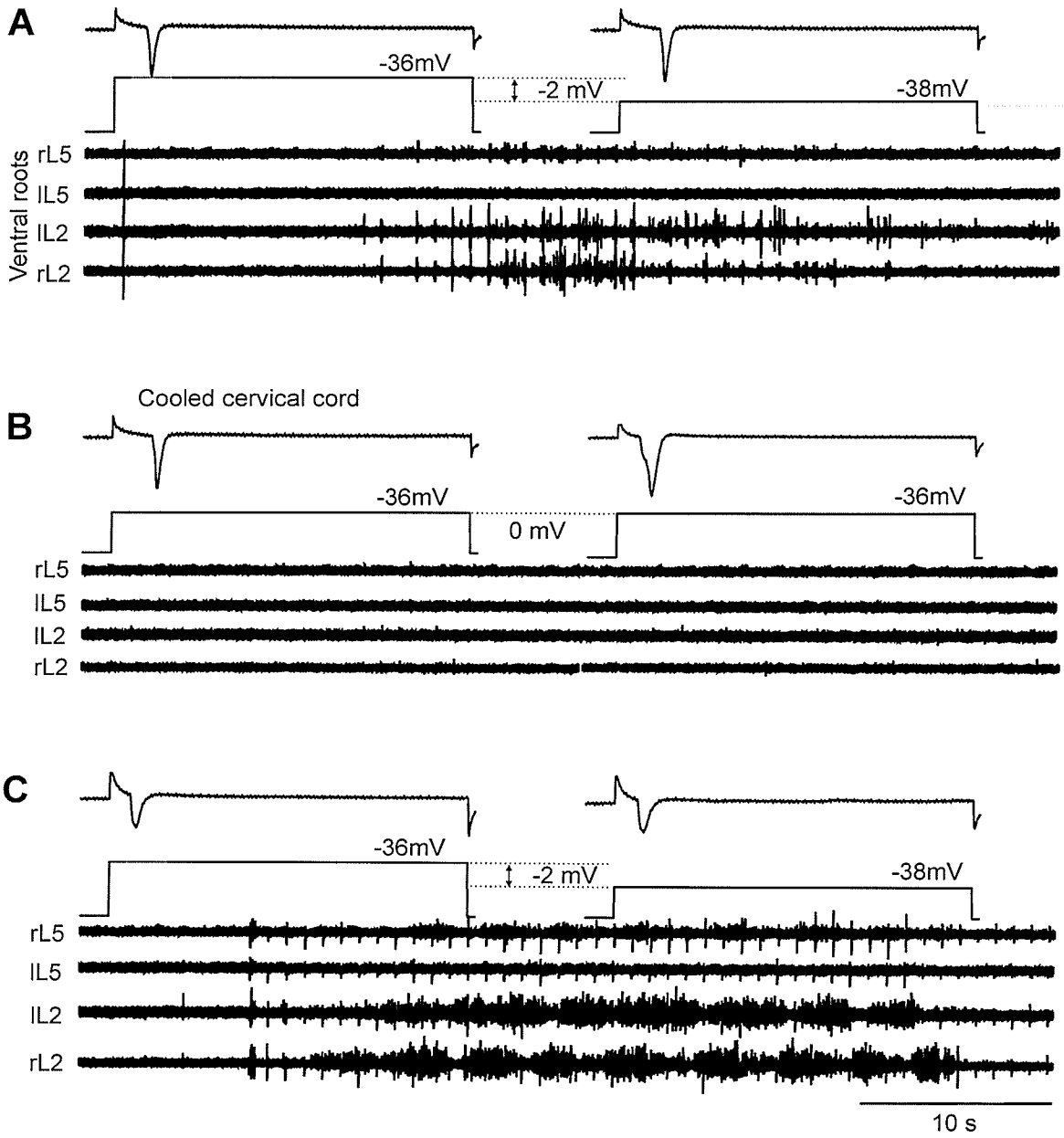

Figure 14
Panel **A** displays the membrane current trace from a lumbar neuron and the 2 mV Vth hyperpolarization during electrial stumulation (1mA, 5ms, 1Hz) of the VMM that induced the VR activity (lower traces). In panel **B** the cervical cord was cooled to 4°C. Note that with the same stimulation parameters as in A there is no change in Vth in the same cell. Notice that the ventral cord output is abolished. Panel **C** shows the same preparation 10 minutes later. The cervical cord has been rewarmed 22°C and the stimulation parameters are the same as in A and B. The Vth hyperpolarization of 2 mV recovers as does the VR activity.

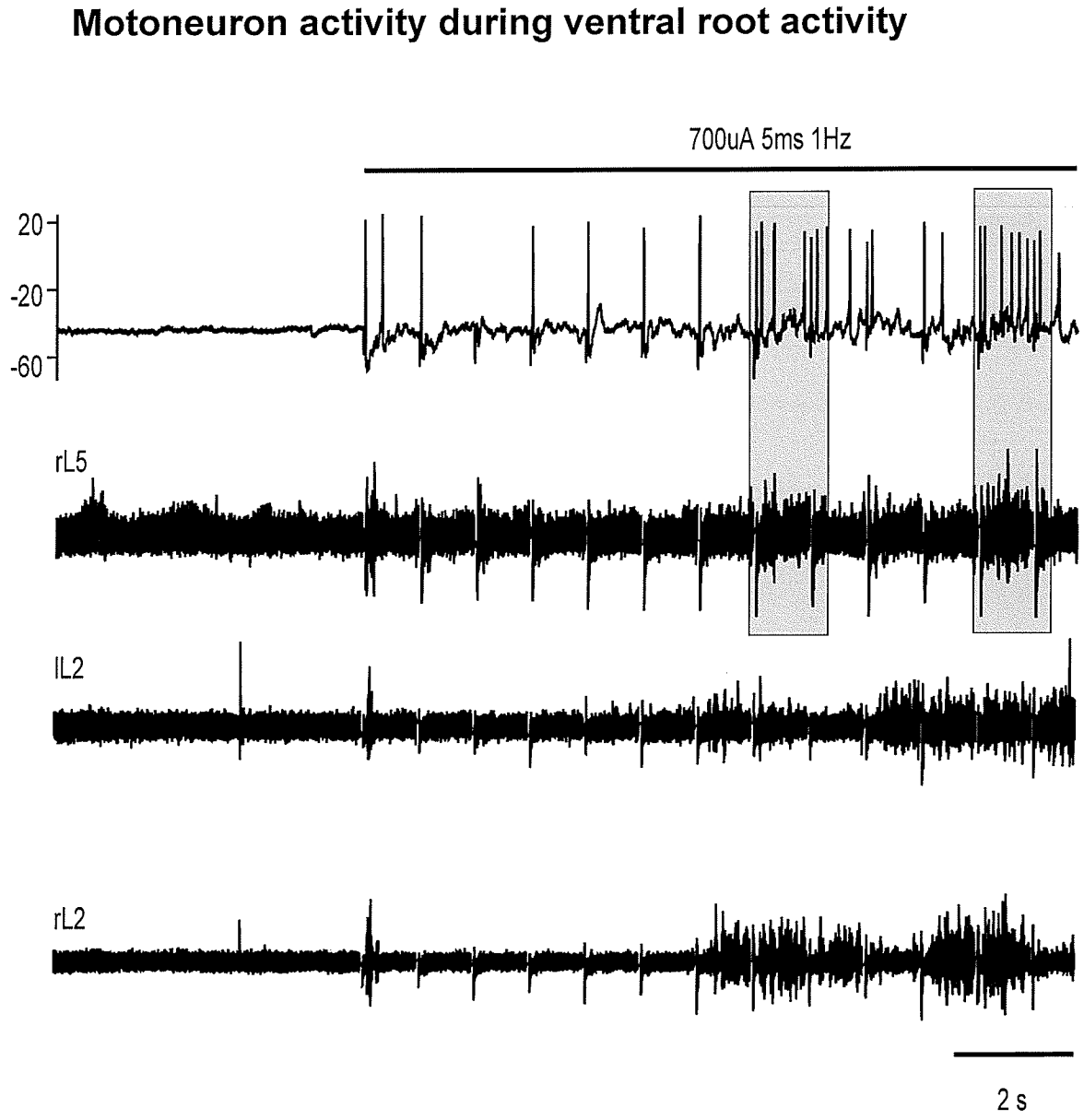

Figure 15
Intracellular current clamp recording of an antidromically indentified motoneruon. Shown below are concurrent ventral root recordings. Ventral root recordings and the intracellular records are silent until electrical stimulation of the VMM begins, causing motoneuron activity and VR activity. Grey boxes indicate areas where ventral root output coincided with motoneuron spiking.

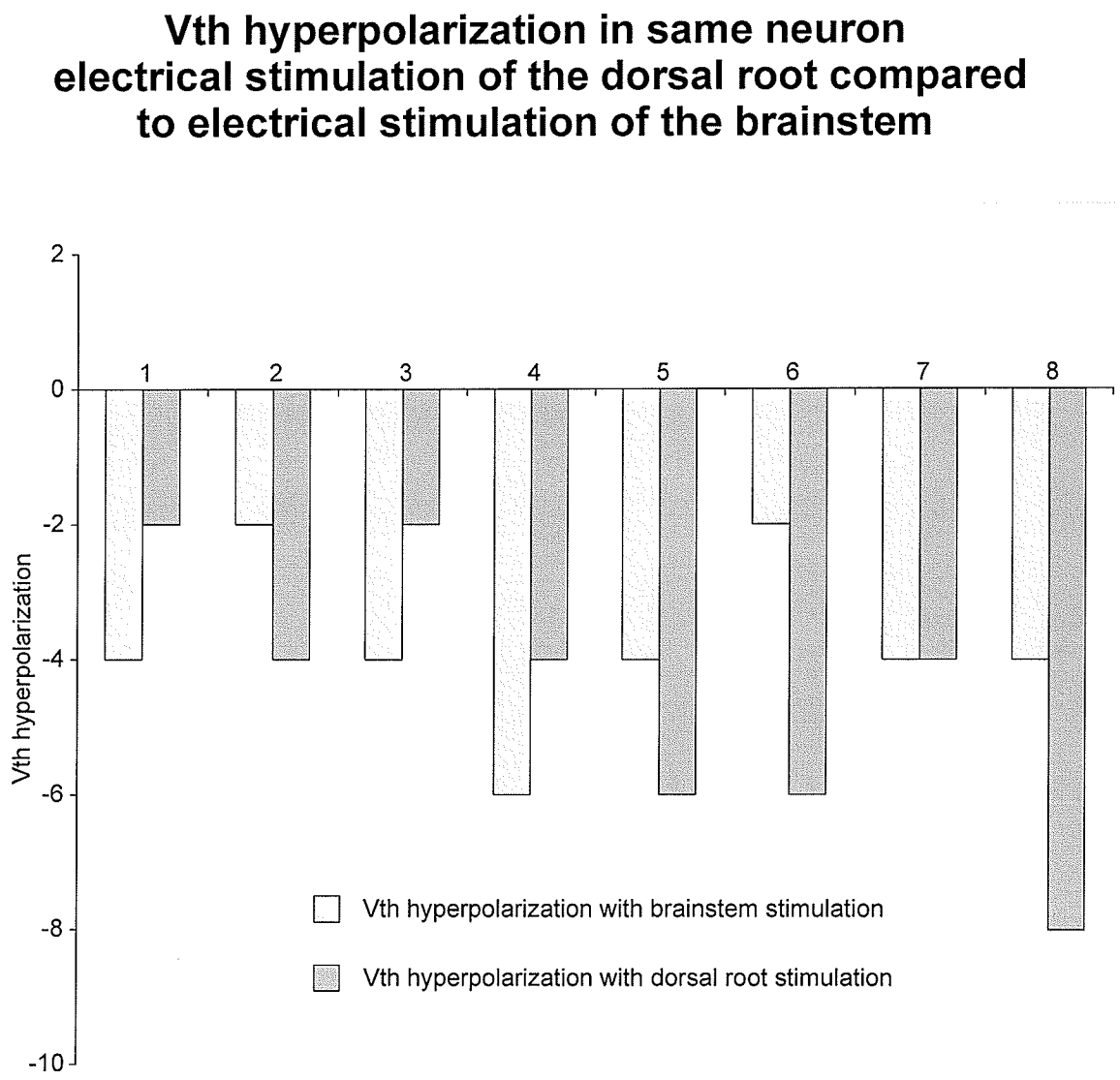

Figure 16
Figure 16 shows the relative amount of Vth hyperpolarization from control in the same neuron when electrical stimulation was delivered to the VMM and subsequently to the rL5 dorsal root. Notice that there is a hyperpolarization of Vth with either stimulation.

Vth hyperpolarization during electrical stimulation of the brainstem compared to bath application of 5-HT

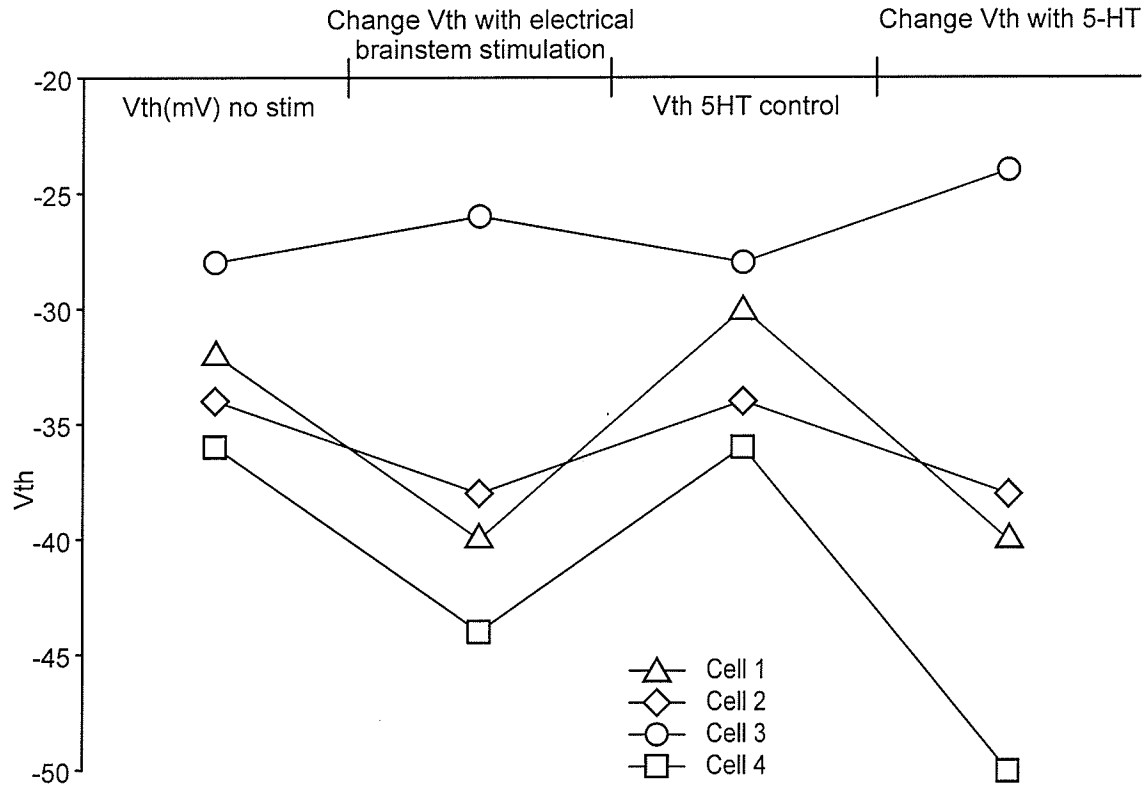

Figure 17
Figure 17 shows a summary of the Vth of 4 cells during 4 different conditions. First shown is the Vth recorded during control, secondly displayed is the Vth recorded during electrical stimulation of the VMM. The cells were allowed to return to control and Vth re-recorded. Thirdly 5-HT (10-40 μM) was added to the bath and caused a hyperpolarization of Vth. In celsl 1 and 2 5-Ht caused the same degree of Vth hyperpolarization as electrical stimulation.

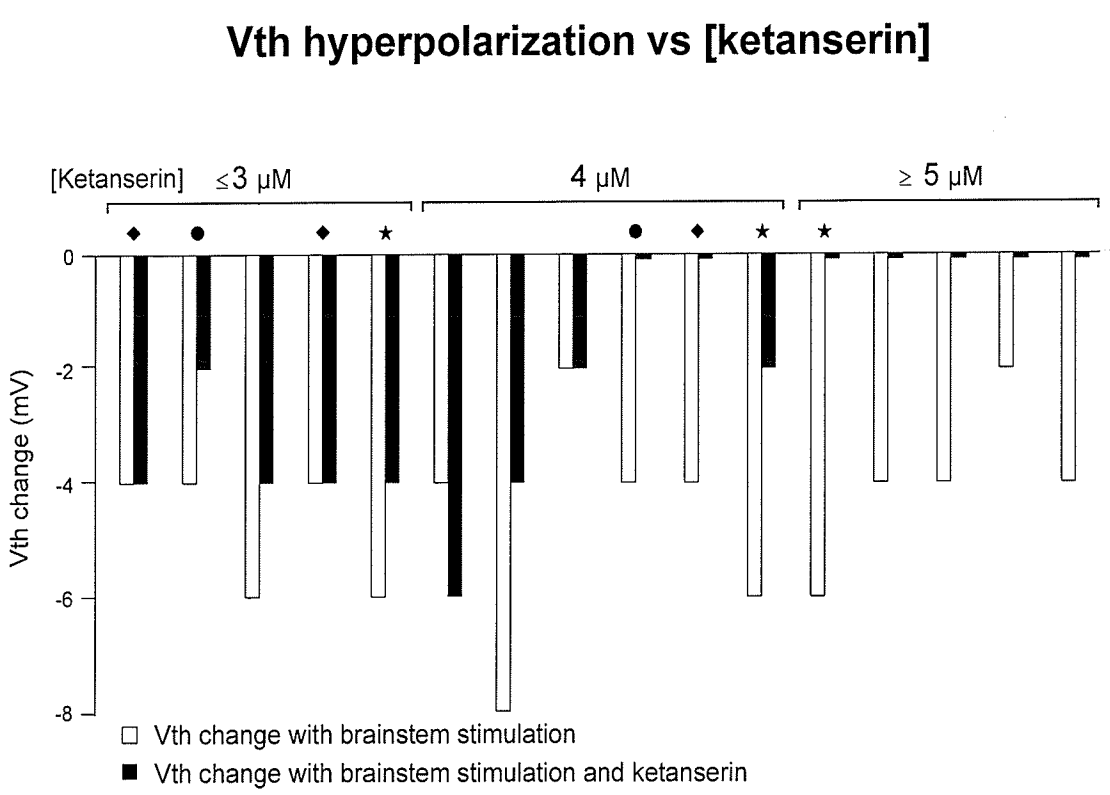

Figure 18
A summary of Vth's from 16 cells where ketanserin , a 5-HT_{2A} antagonist, was added to the lumbar bath. The gray bars indicate how much ketanserin (µM) was added to each cell. The black bars indicate the amount of Vth hyperpolarization with electrical stimulation of the ventromedial medulla before the ketanserin was added. Note: in all cases Vth hyperpolarized with stimulation. The white bars indicate the amount of Vth hyperpolarization once the ketanserin was added to the bath. Instances where there was no Vth change are labelled 0. Note how ketanserin concentrations greater than 6 µM can block the Vth hyperpolarization.

Vth hyperpolarization of repetitive activation of fast Inward Currents

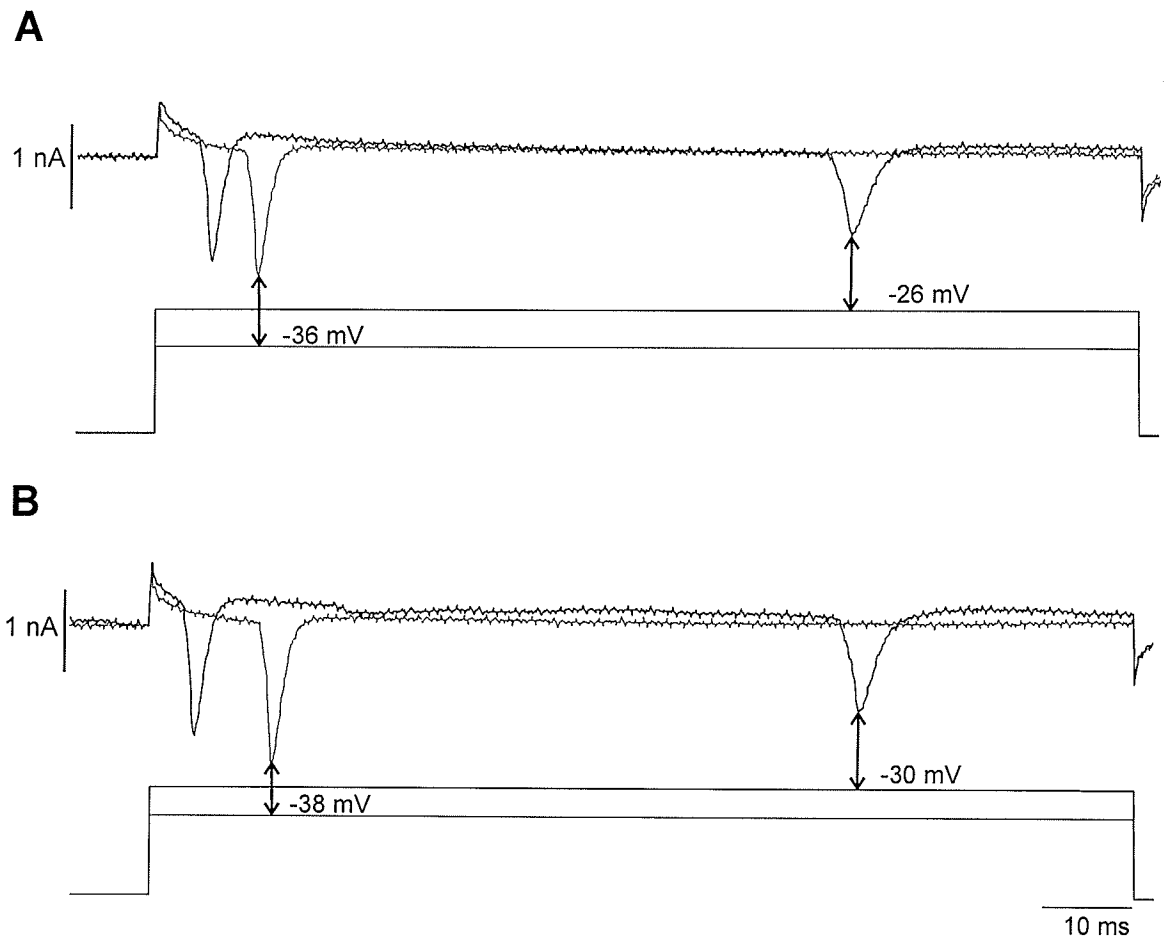

Figure 19
Figure 19 shows the voltage clamp recording froman antidromically identified motoneuron during control and during electrical stimulation of the ventromedial medulla. Figure **A** is the voltage clamp recording during control, displayed is the first fast inward current at 24 mV depolarization, also displayed is the second fast inward current that occured with 34 mV of depolarization. Figure **B** shows the voltage clamp recording from the same motoneuron during stimulation. Notice the first fast inward current Vth hyperpolarized (-2mV) and is evoked with only 22 mV of depolarization. Also notice that the Vth for the second fast inward current also hyperpolarizes (-4mV) vs (-34mV control). This is an example of Vth hyperpolarization of multiple fast inward currents.

Temperature	Chamberpressure (mmHg)								
(°C)	1511	1411	1311	1211	1111	1011	911	811	760
4	26.1	24.4	22.7	20.9	19.2	17.4	15.7	14.0	13.1
5	25.5	23.8	22.1	20.4	18.7	17.0	15.3	13.6	12.8
6	24.8	23.2	21.5	19.9	18.2	16.6	14.9	12.9	12.5
7	24.2	22.6	21.0	19.4	17.8	16.2	14.6	12.6	12.1
8	23.6	22.1	20.5	18.9	17.3	15.8	13.9	12.3	11.8
9	23.1	21.5	20.0	18.5	16.9	15.4	13.5	12.0	11.6
10	22.5	21.0	19.5	18.0	16.5	15.0	13.2	11.8	11.3
11	22.0	20.6	19.1	17.6	16.2	14.7	12.9	11.5	11.0
12	21.5	20.1	18.7	17.2	15.8	14.4	12.6	11.2	10.8
13	21.1	19.7	18.3	16.9	15.5	14.0	12.4	11.0	10.3
14	20.6	19.2	17.9	16.5	15.1	13.7	12.1	10.8	10.1
15	20.2	18.8	17.5	16.1	14.8	13.5	11.8	10.5	9.9
16	19.8	18.4	17.1	15.8	14.5	13.2	11.6	10.3	9.7
17	19.4	18.1	16.8	15.5	14.2	12.9	11.4	10.1	9.5
18	19.0	17.7	16.4	15.2	13.9	12.4	11.1	9.9	9.3
19	18.6	17.4	16.4	14.9	13.6	12.1	10.9	9.7	9.1
20	18.2	17.0	16.1	14.6	13.4	11.9	10.7	9.5	8.9
21	17.9	16.7	15.8	14.3	13.1	11.7	10.5	9.3	8.7
22	17.6	16.4	15.5	14.0	12.9	11.5	10.3	9.1	8.6
23	17.3	16.1	15.2	13.8	12.6	11.3	10.1	9.0	8.4
24	16.9	15.8	14.9	13.5	12.4	11.1	9.9	8.8	8.3

Table 1

In the left column select the appropriate temperature and select the correct pressure across the top of chart, to find the O_2 saturation of aCSF.

At a temperature of 4°C and a pressure of 1511 mmHg (what the aCSF was pressurized to in this experiment) the table shows an oxygen saturation of 26.1 mg O_2 per liter of aCSF.

Normal aCSF is at room temperature and not pressurized, which would result in 8.6 mg O_2 per liter.