AN IN VITRO PROTOCOL FOR RECORDING FROM SPINAL MOTONEURONS OF ADULT RATS

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ABSTRACT

In vitro slice preparations of CNS tissue are invaluable for studying neuronal function. However, up to now, slice protocols for adult mammal spinal motoneurons—the final common pathway for motor behaviors—have been available only for limited portions of the spinal cord. In most cases, these preparations have not been productive due to the poor viability of motoneurons in vitro. This report describes and validates a new slice protocol that for the first time provides reliable intracellular recordings from lumbar motoneurons of adult rats. The key features of this protocol are: pre-exposure to 100% oxygen, laminectomy prior to perfusion, anesthesia with ketamine/xylazine, embedding the spinal cord in agar prior to slicing, and, most important, brief incubation of spinal cord slices in a 30% solution of polyethylene glycol to promote re-sealing of the many motoneuron dendrites cut during sectioning. Together, these new features produce successful recordings in 76% of the experiments and an average action potential amplitude of 76 mV. Motoneuron properties measured in this new slice preparation (i.e., voltage and current thresholds for action potential initiation, input resistance, afterhyperpolarization size and duration, and onset and offset firing rates during current ramps) are comparable to those recorded in vivo. Given the mechanical stability and precise control over the extracellular environment afforded by an in vitro preparation, this new protocol can greatly facilitate electrophysiological and pharmacological study of these uniquely important neurons and other delicate neuronal populations in adult mammals.
INTRODUCTION

Spinal motoneurons were the first central mammalian neurons studied with intracellular electrodes (Brock et al. 1951). Their study has provided the basis for much of what is known about intrinsic neuronal properties and synaptic transmission (Brownstone 2006; Burke 1981, 2006). Furthermore, spinal motoneurons—particularly lumbar and cervical motoneurons—play a central role in motor control as integrators of synaptic input and encoders of motor commands.

Understanding of the essential role of spinal motoneurons would be greatly benefited by acquiring knowledge of their intrinsic properties through the use of in vitro preparations from adult animals. In vitro preparations are widely used in physiological and pharmacological studies of mammalian CNS neurons (Berger 1990; Dingledine et al. 1980; Dunwiddie et al. 1983) because they provide better access, mechanical stability, and control over the extracellular environment than do in vivo preparations. In vitro preparations of immature mammalian spinal cord can provide excellent motoneuron recordings, but suffer from the limitation that the developmental status of the spinal circuitry is different from that in the adult (Biscoe and Duchen 1986; Jiang et al. 1999; Kerkut and Bagust 1995; Rekling et al. 2000). Application of in vitro methodology to the study of spinal motoneurons from adult mammals has had only limited success. The sacro-caudal spinal cord of adult rats affords reliable motoneuron recordings (Bennett et al. 2001), but is not directly relevant to limb motor control. Some in vitro recordings of cervical motoneurons from adult rodents have been reported (Hori et al. 2001, 2002), but in vitro preparations of lumbar spinal cord with viable motoneurons have proven to be
more difficult. A brief report described a small number of recordings from the isolated lumbar spinal cord of the mouse (Fulton 1986), but further studies with that method were not forthcoming. Our own efforts to develop lumbar spinal cord slices using the methodology described for cervical slices (Hori et al. 2001) produced only a few successful recordings (Carp et al. 2003).

The lack of success with in vitro preparations of lumbar spinal cord from adult mammals is largely attributable to two factors: tissue hypoxia/ischemia and mechanical damage. Spinal motoneurons are very sensitive to ischemia and other insults (Carriedo et al. 1996; Nohda et al. 2007; von Lewinski and Keller 2005). Lumbar motoneurons appear to be especially sensitive (Duggal and Lach 2002), which is consistent with their poor viability in vitro (Bagust and Kerkut 1981). In vitro method development to date has focused on rapid cooling to minimize hypoxic and ischemic damage and on using glutamate antagonists or modifications of the extracellular solution to reduce neuronal firing and concomitant excitotoxicity (Bennett et al. 2001; Carlin et al. 2000; Hori et al. 2001, 2002).

While these methods are beneficial, they do not address the mechanical damage that is incurred during tissue preparation. The spinal cord is fragile and easily damaged; dissection techniques try to minimize this damage, e.g., Hori et al. (2001). What has not been addressed previously is the massive damage that sectioning causes by transecting a neuron’s dendrites (Davies et al. 2007). This is particularly severe for spinal motoneurons, which have large cell bodies (e.g., 35-μm average diameter in rats) and elaborate dendritic fields that can extend out 1-2 mm from the soma and constitute ≥96% of their surface area (Chen and Wolpaw 1994; Ulfhake and Kellerth 1981). The many
breaches of the motoneuron membrane produced by dendritic amputations are probably a major reason for the poor viability of motoneurons in slice preparations.

Polyethylene glycol (PEG) induces rapid re-fusion of severed peripheral and central axons (Lore et al. 1999; Shi et al. 1999). We hypothesized that PEG could also re-seal the dendritic processes that are cut during sectioning, and could thereby substantially improve motoneuron viability. This study assesses the impact of PEG and other methodological changes that minimize ischemia and maximize oxygen saturation on production of viable lumbar spinal slices from adult rats. The results show that the combined focus on reducing mechanical damage and maximizing tissue oxygenation greatly facilitate recording from lumbar motoneurons in adult rats. These procedures constitute a new protocol that makes it practical to study these uniquely important CNS neurons in vitro. Portions of this work have been reported in abstract form (Carp et al. 2007).

MATERIALS AND METHODS

Animals and preparation

All animal procedures were in accord with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, DC 1996), and Department of Health, Education and Welfare (DHEW) Publ. No. 0309-
Experiments were conducted in 55 male rats (Sprague-Dawley, 147-513 g, 6-12 weeks for 45 rats whose ages were known) using either the method previously described for preparing cervical spinal slices (Hori et al. 2001) (9 rats) or a new protocol built upon the Hori method (the PEG protocol, described below) (46 rats). For the former experiments, rats were anesthetized with pentobarbital (80 mg/kg i.p.), placed in a supine position, and perfused transcardially for one min with 100 ml of 2-4°C artificial cerebrospinal fluid (aCSF) in which NaCl had been replaced with sucrose (sucrose-aCSF) that contained (in mM): sucrose, 212.5; KCl, 3.5; NaHCO$_3$, 26; MgSO$_4$, 1.3; KH$_2$PO$_4$, 1.2; MgCl$_2$, 2.0; CaCl$_2$, 1.2; and glucose, 10 (298 mOsm). All aCSF solutions were bubbled with 95% O$_2$-5% CO$_2$.

After a dorsal laminectomy was performed, a loop of 5-0 silk suture was placed around the rostral end of the spinal cord and the loose suture ends were attached to a manipulator. While constantly superfusing the spinal cord with 2-4°C sucrose-aCSF, the manipulator was gently raised to facilitate cutting the spinal cord and its roots in a rostral-to-caudal direction. After cutting the suture, the spinal cord was transferred in a plastic spoon filled with cold sucrose-aCSF to a dissection dish filled with 2-4°C sucrose-aCSF. The dura was removed and the spinal roots were cut as close to the cord as possible (long roots can interfere with slicing). The spinal cord was placed in a semicircular channel cut in a block of agar (Sigma, ~2 cm wide x 1.5 cm high x 1 cm thick), and covered with a second agar block. Then the assembly was glued (Loctite 404) to the platform of a vibrating microtome (Vibroslice, Campden or OTS-5000, Frederick Haer) and 450-μm
thick transverse sections were cut in 2-4°C sucrose-aCSF. During slicing, the tissue was advanced at 0.25-0.30 mm/s and the blade (single- or double-edge carbon steel, Feather) oscillation frequency setting was 6-7 (Vibroslice, about 1700-1900 cycles per min) or 9-10 (OTS-5000).

The slices were incubated for one hr at 34-36°C in normal-sodium aCSF, which consisted of (in mM): NaCl, 125; KCl, 3.5; NaHCO₃, 26; MgSO₄, 1.3; KH₂PO₄, 1.2; CaCl₂, 2.4; and glucose, 10 (294 mOsm). The slices were then transferred to mesh-floored holding chambers with normal aCSF at 29-31°C and remained there until used for recordings.

The PEG protocol is based on the preceding protocol, but has the following modifications. (1) The rats were anesthetized with ketamine:xylazine (80:10 mg/kg, i.p.). (2) The laminectomy was performed before (rather than after) the transcardiac perfusion. (3) The rats received 100% oxygen by mask throughout the laminectomy and continuing up until the transcardiac perfusion. (4) Just prior to perfusion, the rat was carefully turned from a prone to a supine position, and the exposed spinal cord was positioned over a small well in the dissection surface filled with 2-4°C sucrose-aCSF to begin chilling the spinal cord and protect it from mechanical damage. (5) In preparation for slicing, the spinal cord was removed from the dissection dish, blotted on a pre-moistened piece of filter paper, and suspended within a short glass cylinder (13 mm high x 7 mm i.d.) in a 2-4°C sucrose-aCSF-filled stainless steel bath) while warm low-gelling-temperature agarose (~28°C, Sigma Type IX-A, 6% in sucrose-aCSF) was injected around it with a 1-cc syringe. The glass cylinder was removed from the bath after the agar hardened (~15 s). The agarose-embedded cord was partially ejected by applying gentle pressure with a one-
cc syringe plunger to allow excess agarose to be trimmed with a razor blade to within 1 mm of the cord, and then completely ejected directly onto a small drop (~10-12 µl) of glue (Loctite 404) on the mounting platform of the vibrating microtome. A 14-mm high by 10-mm wide Teflon block at the end of the mounting platform opposite the microtome blade prevented the agarose (which was glued directly in front of it) from bending or being dislodged during cutting. (6) Immediately after cutting, each slice was incubated for 60 s in PEG 2000 (30% w/v in distilled water, 870 mOsm; Sigma) (with fresh solution for each slice using 12-well tissue culture trays) and was then rinsed for one min in normal aCSF at 34-36°C twice immediately prior to a one-hr incubation in normal aCSF at 34-36°C. The choice of a 60-s PEG incubation was guided by preliminary studies in 9 animals (rats and mice) that failed to produce any acceptable recordings using 45-s, 90-s, or 120-s exposures (although acceptable recordings were obtained in two of three mouse experiments using a 75-s incubation).

Data collection and analysis

Each slice to be studied was transferred to the recording chamber, held on a mesh platform by harp-shaped silver wires with elastic threads, and superfused with normal aCSF at 3 ml/min at 29-31°C. Intracellular impalements were made with 30-60 MΩ long-taper electrodes or with 9-20 MΩ rapid-taper (“bee-stinger”) electrodes filled with 3-M potassium acetate. Data were recorded (Dagan 8700), low-pass filtered at 3-10 KHz, sampled at 6-20 KHz, and stored using WinWCP (Strathclyde Electrophysiology Software), and were analyzed using custom software. Impalements typically lasted 10-20
min (median recording time after stabilization was 9 min, with 10% of cells recorded for 20-90 min).

Hyperpolarizing 40-ms current pulses were used to evaluate input resistance and membrane time constant. Action potentials were elicited by short (0.6- to 1.0-ms) depolarizing current pulses (Figure 2B, left panels) and/or by antidromic activation by stimulation with a Teflon-insulated silver wire electrode (0.125 mm diameter, ~100 µm exposed tip) placed on the ventral root stub (Figure 2A). Action potential amplitude was calculated as the difference between the peak of the action potential and the average resting potential during the five ms prior to stimulation. AHP duration, amplitude, and half-time (i.e., time from AHP maximum amplitude until recovery to half-maximal amplitude) were determined from action potentials elicited by the short current pulses. Long (40-ms) depolarizing current pulses were used to determine rheobase (i.e., current at which probability of firing is 50%). The difference in amplitude between action potentials elicited by long and short depolarizing pulses determined the threshold depolarization (i.e., the depolarization from resting potential needed to reach the voltage threshold for action potential initiation (Carp 1992; Gustafsson and Pinter 1984); see Figure 2B). Voltage threshold was calculated as the sum of the depolarization threshold and the resting membrane potential. Slow triangular current ramps (0.2-2.0 nA/s for 5 s in each direction) were used to induce repetitive firing. Repetitive firing behavior was characterized by the instantaneous firing rates and the currents at the onset and offset of repetitive firing.

Motoneuron labeling
Two rats received intramuscular injections of cholera toxin subunit-B conjugated to Alexa 594 and/or to FITC (Invitrogen; 1-2 µg/µl solution in sterile saline) to retrogradely label gastrocnemius and soleus motoneuron pools. Under ketamine-xylazine anesthesia (as described above), the skin of the lateral aspects of the lower hindlimbs was incised and the lateral and medial gastrocnemius and soleus muscles were exposed. Using a 100-µl syringe with a 31-ga needle under direct observation with a dissection microscope, the gastrocnemius muscles were injected slowly with 50-110 µg of label divided among three injection tracks and soleus muscles were injected with 20-40 µg of label divided between two injection tracks. After injection, the needle was withdrawn gradually over three min. After the injection site and surrounding tissues were flushed thoroughly with sterile saline, the incision was infiltrated with bupivicaine, closed with wound clips and dressed with nitrofurazone. Three or four days after injection, spinal slices were prepared according to the PEG protocol.

Statistics

Statistical analyses of continuous variables used the $t$-test. Analysis of preparation success vs. failure used the Maximum Likelihood Chi-square test.

RESULTS

Study design and data pool
Intracellular current clamp recordings were made with sharp electrodes in transverse slices of lumbar spinal cord of adult rats from 105 neurons with action potential amplitudes $\geq 60$ mV, which was the minimum value for acceptance into the data pool. Nine of them were tested by antidromic activation and were positively identified as motoneurons (see example in Figure 2A). The rest of these neurons were presumed to be motoneurons because: (1) they were located in dark patches of the ventrolateral gray matter consistent with motor columns, (2) they had properties similar to those of the antidromically identified motoneurons (e.g., input resistance=$7.3\pm3.8$ vs. $7.8\pm4.9$ $\Omega$ and rheobase=$2.9\pm0.8$ vs. $3.1\pm2.6$ nA for antidromic motoneurons vs. other neurons, respectively), and (3) their input resistances were all substantially lower and their rheobases were all higher than the expected range of values for spinal interneurons capable of repetitive firing (Theiss and Heckman 2005).

Three measures were used to evaluate the success of each preparation: (1) the percentage of experiments that were successful, i.e., yielding at least one motoneuron meeting the acceptance criterion; (2) the average number of accepted motoneurons studied per experiment; and (3) the average action potential amplitude of the accepted motoneurons.

Motoneuron viability with the PEG protocol

The PEG protocol reliably produced slices with viable motoneurons. Recordings were attempted in a median of four slices per preparation. Neuronal impalements were
obtained in 56% of the slices in which recording was attempted. The PEG protocol produced viable slices in 42 out of 46 preparations (96%; three to five viable slices in 11 preparations, two in 17 preparations, and one in 14 preparations). It produced at least one acceptable (i.e., >60-mV action potential) motoneuron recording per preparation in 35 out of 46 preparations (76%). The average number of acceptable motoneurons recorded per preparation was 2.15 (±0.30 SE) and the average action potential amplitude per successful preparation was 75.7 (±1.1) mV. These results clearly demonstrate the reliability, yield, and recording quality achieved by the PEG protocol.

In contrast, application of the previously reported method for cervical spinal cord slices (Hori et al. 2001) resulted in successful recordings in 2 out of 9 experiments (22%; different from PEG protocol, P=0.009 by Chi-square test), and an average of 0.44 (±0.29 SE) recordings per experiment (different from PEG protocol; p=0.0016 by t-test) with an average action potential amplitude of 61.3 (±1.3) mV (different from PEG protocol, P=0.016 by t-test). These action potential amplitudes are comparable to those from some recordings illustrated in Hori et al. (2001).

Factors affecting success

For the PEG protocol studies, the dissection time (i.e., the time from starting the thoracotomy until the first slice was cut) of successful preparations (range 8.4-12.5 min, median 11.0 min for 13 preparations in which this was measured) was not significantly different from that of unsuccessful preparations (range 10.3-21.0 min, median 11.5 min; P=0.27, Wilcoxon Rank Sum test). Also, dissection time did not linearly predict the
number of motoneurons studied per preparation (P=0.45 by linear regression). Still, it should be noted that all preparations with ≥three motoneurons studied had dissection times of ≤12.5 min.

Preparation success did not depend on body weight or age. Body weight (which was used as a covariant of age because data on animal age was incomplete) did not account for a significant amount of the variation in the outcome variables (Least-squares fit, P>0.9 for weight for both the number of motoneurons studied per preparation and their average action potential amplitude).

Results with the PEG protocol improved with repeated performance. Only six out of the first 10 preparations were successful, while all of the final 10 preparations were successful (P=0.01 by Chi-square test). The average number of motoneurons recorded per preparation increased from 1.20 (±0.49 SE) for the first 10 preparations to 2.50 (±0.27) for the last 10 preparations (P=0.03 by t-test). Average action potential amplitude per successful preparation increased from 69.1 (±2.5) mV for the first 10 preparations to 76.2 (±1.4) mV for the last 10 preparations (P=0.02 by t-test)

Motoneuron location and identity

Using the PEG protocol, recordings were made from 101 lumbar motoneurons, of which 64 were putative pudendal motoneurons and 37 were putative hindlimb motoneurons. The putative pudendal motoneurons were recorded from characteristic round dark regions in the lateral grey matter of the ventral horn (arrows in Figure 1A). These areas probably represent dendritic bundles and cell bodies of pudendal
motoneurons in caudal L5-L6 that innervate external urethral sphincter muscles (and ischiocavernosus in males) (McKenna and Nadelhaft 1986; Schroder 1980).

The putative hindlimb motoneurons were typically recorded from 1-8 slices (0.45-3.6 mm) rostral to the pudendal motoneuron slices. They were located throughout the motor columns of the ventral horn, which are less intensely dark and more irregularly shaped (indicated by dotted outlines in Figure 1B) than the pudendal motoneuron region. Their location rostral to the putative pudendal motoneurons indicates that they are mainly L4-5 hindlimb motoneurons (Nicolopoulos-Stournaras and Iles 1983). This identification is also supported by observations in two rats in which gastrocnemius and soleus muscles were injected with fluorescently labeled cholera toxin. The labeled motoneurons were concentrated in the same central and lateral ventral grey matter areas from which the putative hindlimb motoneurons were recorded. No labeled motoneurons were found in the more caudal location of the putative pudendal motoneurons.

Four additional motoneurons were recorded from cervical slices in the 9 preparations that used the protocol of Hori et al. (2001). The recordings targeted the small grey irregularly shaped clusters in the mid- to lateral-ventral horn that form the motoneuron columns of forelimb and phrenic motoneurons.

**Motoneuron properties**

From the 101 lumbar motoneurons with action potentials ≥60 mV, we recorded responses of 92 motoneurons to a series of rectangular current pulse injection protocols for determination of input resistance, time constant, current threshold (rheobase), and
voltage threshold, and to triangular current ramps for determination of repetitive firing properties.

Table 1 summarizes the properties of hindlimb and pudendal motoneurons. Hindlimb motoneurons have lower input resistance and higher rheobase than do pudendal motoneurons, which is consistent with the fact that pudendal motoneurons are smaller (Chen and Wolpaw 1994; Collins et al. 1992). Resting potential is more hyperpolarized in hindlimb than in pudendal motoneurons. These relationships are unlikely to reflect differences in impalement quality (since input resistance is lowest in hindlimb motoneurons, which have the largest action potentials and most negative resting potentials), and thus these observations probably reflect true physiological differences between hindlimb and pudendal motoneurons.

Table 2 compares the properties of rat hindlimb motoneurons recorded *in vitro* (i.e., present data) and *in vivo* (Button et al. 2006). Motoneurons recorded *in vivo* (right-hand columns of Table 2) display a systematic distribution of properties in which those innervating fast-twitch muscle (fast motoneurons) tend to have higher rheobase, lower input resistance, and shorter AHP duration than do those innervating slow-twitch muscle (slow motoneurons) (Burke 1981). The same pattern is seen *in vitro*, where fast motoneurons (distinguished from slow motoneurons by their AHP half-time (Gardiner 1993)) have significantly lower rheobases, higher input resistances, and smaller AHP amplitudes than slow motoneurons.

When triangular current ramps were injected, motoneurons recorded *in vitro* behaved like motoneurons recorded *in vivo* (Button et al. 2006): they were recruited during the ascending current ramp, fired repetitively, and then were derecruited during
the descending current ramp (Figure 3). Firing behavior was quantified by the instantaneous firing rates (calculated from the interspike intervals) and the currents at the onset and offset of firing. Ramp onset and offset firing rates and currents were significantly higher in fast than in slow motoneurons in vitro, as they are in vivo (Table 2). The difference between offset and onset currents was significantly different between fast and slow motoneurons in vitro, but not in vivo (although the latter displayed a similar trend).

During triangular current ramps, all motoneurons recorded in vitro exhibited one of two characteristic response patterns. One pattern was higher offset firing rate and offset current than onset firing rate and onset current (i.e., offset minus onset is positive; Figure 3A, C, E, and G). The other pattern was lower offset firing rate and current than onset firing rate and current (i.e., offset minus onset current is negative; Figure 2B, D, F, and H). These response patterns are comparable to the “adaptive” and “linear-plus-sustained” firing patterns, respectively, described for rat motoneurons in vivo (Button et al. 2006). The linear-plus-sustained pattern is more frequently expressed in slow (8/14 (57%)) than in fast (7/28 (25%)) hindlimb motoneurons recorded in vitro (P=0.04, Chi-square test).

DISCUSSION

This report describes a new protocol for performing intracellular recording from motoneurons in lumbar transverse spinal slices from adult rats. The five features of the PEG protocol that distinguish it from the methodology upon which it is based (Hori et al.
are: (1) incubation of slices in PEG, (2) performing the laminectomy prior to transcardiac perfusion, (3) exposure to 100% oxygen prior to transcardiac perfusion, (4) anesthesia with ketamine/xylazine, and (5) embedding the spinal cord in agar before slicing. Together, these procedures routinely produced lumbar spinal slices in which motoneurons were recorded with action potentials of 60-90 mV.

The validity of the PEG protocol is indicated by the close similarities between the motoneuron data it has produced and data from motoneurons studied in vivo. These similarities include: (1) hindlimb motoneurons have lower input resistance and higher rheobase than pudendal motoneurons in both in vivo data (Burke 1981; Hochman et al. 1991; Sasaki 1991) and our in vitro data; (2) rat motoneurons display comparable patterns of type-dependent (i.e., fast vs. slow) differences in rheobase, input resistance, AHP amplitude, and repetitive firing onset and offset rates and currents in both in vivo data (Burke 1981; Button et al. 2006) and our in vitro data; (3) the two dominant types of motoneuron repetitive firing behavior (i.e., adaptive and linear-plus-sustained firing) seen in our in vitro data (Figure 3) are also seen in previously reported in vivo data (Button et al. 2006); (4) the linear-plus-sustained repetitive firing behavior we found in vitro probably reflects a persistent inward current that is well characterized in motoneurons in vivo (Heckmann et al. 2005); and (5) firing patterns indicative of an underlying inward persistent current are more frequently evident in slow than in fast motoneurons in both in vivo data (Button et al. 2006) and our in vitro data.

The modest quantitative differences in rat lumbar motoneuron properties between in vivo data (Button et al. 2006) and our in vitro data probably reflect differences in synaptic inputs to the motoneurons rather than differences in motoneuron intrinsic
properties. That input resistance is lower and rheobase is higher \textit{in vivo} than \textit{in vitro} is unlikely to reflect a lower impalement quality (due to the mechanical instability inherent to \textit{in vivo} recording), because action potential amplitude and resting potential (which should also be adversely affected by poor impalements) are comparable \textit{in vivo} and \textit{in vitro} (Table 2). Rather, these observations probably reflect greater synaptic drive to motoneurons \textit{in vivo} than \textit{in vitro}. Similarly, the observation that rat motoneurons display lower firing rates and less firing-rate hysteresis during ascending and descending current ramps \textit{in vitro} than \textit{in vivo} (Button et al. 2006) probably reflects the reduction \textit{in vitro} of neuromodulatory influences on the persistent inward currents that control repetitive firing behavior (Heckmann et al. 2005).

Although the PEG protocol produces recordings from lumbar motoneurons \textit{in vitro} with action potentials amplitudes comparable to those \textit{in vivo} (Table 2; Beaumont and Gardiner 2003; Cormery et al. 2005), Harvey et al. (2006) report larger action potentials recorded from sacro-caudal motoneurons \textit{in vitro} (i.e., average action potential amplitude of 92.0 mV and average overshoot of 19.3 mV). This difference could reflect an intrinsic difference between lumbar and sacro-caudal motoneurons. It could also reflect the deleterious effect of slicing (the sacro-caudal preparation is isolated, but unsectioned) and/or differences in viability \textit{in vitro} between lumbar and sacro-caudal motoneurons. In any case, PEG protocol might be further improved. Possible modifications include parasagittal (instead of transverse) slicing to take advantage of the motoneurons’ smaller medio-lateral than rostro-caudal dendritic extent (Chen and Wolpaw 1994), replacing the warm agarose embedding medium with a cold photocured hydrogel to reduce spinal cord heat exposure, and preconditioning to improve
motoneuron resistance to hypoxia and ischemia (Sharp et al. 2004; Steiger and Hanggi 2007).

Consideration of the mechanisms of the key features of the PEG protocol provides insight into the efficacy of their addition to the slice preparation protocol. PEG fuses lipid membranes by excluding water from the lipid interface (Lentz 2007). It fuses the cut ends of peripheral nerves and re-establishes axonal conduction (Lore et al. 1999; Shi et al. 1999), and in a model of spinal cord injury it protects central neurons by making them less leaky (Shi and Borgens 2000). In the present context, PEG may fuse freshly transected dendrites at the slice surfaces, thereby preventing the ionic imbalance that would otherwise result from these large transmembrane shunts. If this hypothesis is correct, PEG application is the only treatment that directly promotes sealing of the neuronal elements cut during slice preparation. In addition, PEG incubation could be incorporated into slice protocols for other CNS tissue in which the target neuron’s extensive dendritic arborization puts it at risk of damage during sectioning (e.g., Davies et al. 2007).

Some of the other distinctive features of the PEG protocol address the motoneuron’s responses to the ischemic/hypoxic insult (Acker and Acker 2004; Krnjevic 1999) that begins at the onset of the thoracotomy immediately before transcardiac perfusion. Prior exposure to 100% oxygen (as used in an in vitro preparation of the sacro-caudal spinal cord (Li and Bennett 2003)) maximizes tissue oxygen saturation (which may be compromised by anesthesia). Performing the laminectomy prior to the perfusion shortens by at least two minutes the period of hypoxia/ischemia that occurs before the spinal cord is fully cooled. Additionally, performing the laminectomy prior to the
perfusion allows the laminectomy to be performed more slowly, thereby reducing the risk of damaging the spinal cord. Anesthesia with the NMDA receptor antagonist ketamine may reduce glutamatergic excitotoxic damage (Albensi 2007).

Embedding the spinal cord in warm agar followed by rapid cooling may help to further reduce mechanical trauma. Compared to using agar blocks (which require a precisely cut channel in which the spinal cord rests during slicing) (Hori et al. 2001), spinal cord embedding does not require manual shaping of the agar, reduces the potential for damage by tissue compression, and surrounds the spinal cord completely, thereby providing integral support and decreasing the chance of the spinal cord being pulled out of the agar during slicing.

Our experimental design tested the efficacy of the PEG protocol as a whole, but did not assess the individual contributions of its components. Except for PEG, all of the other individual components have been used in other *in vitro* preparations (Carlin et al. 2000; Hirayama et al. 1988; Li and Bennett 2003; Takahashi 1978), and they presumably contribute to the efficacy of those protocols and of the current PEG protocol. Nevertheless, the fact that a protocol containing PEG is the only method described to date that produces reliable motoneuron recordings in lumbar slices from adult rats and the observation that its effect on slice viability depends on exposure time suggest that PEG makes the most important neuroprotective contribution to the protocol.

In summary, this study describes and validates a new protocol for studying lumbar motoneurons in spinal cord slices from adult rats. Ancillary studies indicate that this protocol is also applicable to adult mouse spinal cord (Carp et al. 2007). Rats and mice have rapidly become the models of choice for studies of normal and pathological
motor function. The reliable *in vitro* preparation described here should foster physiological and pharmacological *in vitro* studies of motoneuron intrinsic properties and synaptic inputs. This new protocol should be particularly valuable for studying motoneuron plasticity during aging, after ischemic or traumatic injury to the brain or spinal cord, and in animal models of neurodegeneration.
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REFERENCES


Cormery B, Beaumont E, Csukly K, Gardiner P. Hindlimb unweighting for 2 weeks alters


FIGURE LEGENDS

Figure 1. Transverse 450-µm-thick slices from the spinal cord 6.8 mm caudal (A) and 3.7 mm caudal (B) to the widest part of the lumbar enlargement of a 190-g (~six-week old) rat. The more caudal slice (A) contains a bilateral pair of dark circular regions (arrows) in the ventral horns (ventral towards bottom of picture) that correspond to the dorsolateral cell group in L5-L6 that contains pudendal motoneurons (McKenna and Nadelhaft 1986; Schroder 1980). This cell group is evident (but less prominent) in one slice rostral and two slices caudal to the one shown, but not beyond this region. In more rostral slices (e.g., B), irregularly shaped and usually less intensely dark motoneuron columns are evident throughout the ventral horn (circumscribed by dashed lines). Motoneurons recorded in these L4-5 regions are likely to innervate hindlimb muscles.

Figure 2. Examples of action potentials elicited in motoneurons. (A) Membrane potential traces show that antidromic stimulation of the ventral root at just-threshold intensity elicits (solid line) or fails to elicit (dashed line) an action potential. Resting potential, -61.7 mV). (B) Membrane potential (upper traces) recorded during current injections (lower traces) for same motoneuron as in panel A (voltage traces in panels A and B are shown with the same absolute voltage level and scaling factor). Starting from similar resting potentials (-61.7 mV (left) and -61.3 mV (right); average membrane potential shown as lower dashed horizontal line), just-threshold short (left) or long (right) depolarizing current pulses elicit action potentials with peaks that are similar to each other (upper horizontal dashed line) and to that of the antidromic action potential in panel
A. The amplitude of each action potential is indicated by vertical double-headed arrows. The difference between these two amplitudes defines the threshold depolarization (i.e., depolarization from the resting potential required to reach the voltage threshold for action potential initiation).

Figure 3. Examples of two types of repetitive firing behavior in response to injected current. Membrane potential (MP) (A, B) and instantaneous firing rate in pulses per s (pps) (C, D) are shown during triangular ramp current injections (E, F) as a function of time. Dashed vertical arrows indicate recruitment and derecruitment times, and dashed horizontal lines indicate recruitment and derecruitment current levels (E, F). A DC bias current of +5.6 nA is applied to the motoneuron on the left and +1.9 nA to the motoneuron on the right throughout the triangular ramp current (not included in current traces in E and F). The current-rate relationships (G, H) reveal the time course of the change in instantaneous firing rate during the rising and falling legs of the triangular ramp current (firing sequence starting at recruitment and ending at derecruitment indicated by upward-pointing and downward-pointing arrows, respectively). The left-hand panels (A, C, E, and G) illustrate a type of firing behavior in which the current and firing rate at which this motoneuron is recruited are lower than the current and firing rate at which it is derecruited. This is expressed as clockwise hysteresis (as indicated by the arrows) in the current-rate plot (G). The right-hand panels (B, D, F, and H) illustrate a different type of firing behavior in which the current and firing rate at which this motoneuron is recruited are higher than the current and firing rate at which it is
derecruited. This is expressed as a counter-clockwise hysteresis at the beginning and end (arrows) of the current-rate plot (H).
Figure 2
Figure 3
Table 1. Properties of hindlimb and pudendal motoneurons

<table>
<thead>
<tr>
<th>Property</th>
<th>Hindlimb(^a) (mean ± SD)</th>
<th>Pudendal(^a) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential (mV)</td>
<td>-66.9±12.3</td>
<td>-59.8±9.2**</td>
</tr>
<tr>
<td>AP amplitude(^b) (mV)</td>
<td>78.7±8.9</td>
<td>77.2±7.9</td>
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<tr>
<td>Input resistance (MΩ)</td>
<td>5.1±3.4</td>
<td>9.2±4.8***</td>
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<tr>
<td>Time constant (ms)</td>
<td>3.6±2.3</td>
<td>4.4±2.0</td>
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<tr>
<td>AHP duration (ms)</td>
<td>45.5±12.8</td>
<td>47.6±11.2</td>
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<tr>
<td>AHP half-time (ms)</td>
<td>17.2±7.0</td>
<td>18.6±5.8</td>
</tr>
<tr>
<td>AHP amplitude (mV)</td>
<td>-3.0±2.0</td>
<td>-4.3±3.1*</td>
</tr>
<tr>
<td>Rheobase (nA)</td>
<td>4.3±3.0</td>
<td>2.4±1.9**</td>
</tr>
<tr>
<td>Threshold depolarization(^c) (mV)</td>
<td>18.2±7.5</td>
<td>17.5±6.5</td>
</tr>
<tr>
<td>Number of cells</td>
<td>32-35</td>
<td>49-57</td>
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</tbody>
</table>

\(^a\)See text for motoneuron identification criteria. \(^b\)Amplitude of action potential elicited by brief (0.4-1.3-ms (typically 0.6-ms) current pulse). \(^c\)Depolarization from resting potential required to elicit action potential. *P<0.05, **P<0.01 and ***P<0.001, different from hindlimb motoneurons by \(t\)-test.
<table>
<thead>
<tr>
<th>Property</th>
<th>In vitro (mean ± SD)</th>
<th>In vivo&lt;sup&gt;a&lt;/sup&gt; (mean ± SD)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Fast&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Slow&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Resting potential (mV)</td>
<td>-68±11</td>
<td>-63±14</td>
</tr>
<tr>
<td>Action potential amplitude (mV)</td>
<td>79.9±8.9</td>
<td>75.7±8.6</td>
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<tr>
<td>Input resistance (MΩ)</td>
<td>4.0±2.1**</td>
<td>7.7±4.8</td>
</tr>
<tr>
<td>AHP half-time (ms)</td>
<td>14±3***</td>
<td>27±4</td>
</tr>
<tr>
<td>AHP amplitude (mV)</td>
<td>-2.4±1.2*</td>
<td>-4.5±2.8</td>
</tr>
<tr>
<td>Rheobase&lt;sup&gt;d&lt;/sup&gt; (nA)</td>
<td>4.9±3.2*</td>
<td>2.8±1.5</td>
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<tr>
<td>Voltage threshold (mV)</td>
<td>-51±12</td>
<td>-46±9</td>
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<tr>
<td>Ramp onset firing rate&lt;sup&gt;e&lt;/sup&gt; (pps)</td>
<td>16.7±6.6**</td>
<td>9.2±1.1</td>
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<tr>
<td>Ramp offset firing rate&lt;sup&gt;f&lt;/sup&gt; (pps)</td>
<td>12.5±3.6*</td>
<td>8.8±2.6</td>
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<tr>
<td>Ramp onset firing current&lt;sup&gt;g&lt;/sup&gt; (nA)</td>
<td>7.9±4.6**</td>
<td>2.8±1.8</td>
</tr>
<tr>
<td>Ramp offset firing current&lt;sup&gt;h&lt;/sup&gt; (nA)</td>
<td>8.2±4.9**</td>
<td>2.5±1.6</td>
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<tr>
<td>Offset-onset current&lt;sup&gt;i&lt;/sup&gt; (nA)</td>
<td>0.3±0.8***</td>
<td>-0.3±0.2</td>
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<tr>
<td>Number of motoneurons</td>
<td>15-26</td>
<td>6-10</td>
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</table>
Data from Table 1 of Button et al. (2006), except for action potential amplitude data, which are calculated from 216 motoneurons pooled from Table 1 of Beaumont and Gardiner (2003) and Table 1 of Cormery et al. (2005). Fast motoneurons defined by AHP half-time <20 ms; asterisks indicate significant differences from slow motoneurons at *P<0.05, **P<0.01, and ***P<0.001. Slow motoneurons defined by AHP half-time ≥20 ms. Current threshold for action potential initiation during 40-ms depolarizing pulse. Instantaneous firing rate from first interspike interval during ascending current ramp. Instantaneous firing rate from last interspike interval during descending current ramp. Current during ascending ramp at which firing begins. Current during descending ramp at which firing ends. Ramp current at firing offset minus ramp current at firing onset.