An in vitro spinal cord slice preparation for recording from lumbar motoneurons of the adult mouse

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Mitra P, Brownstone RM. An in vitro spinal cord slice preparation for recording from lumbar motoneurons of the adult mouse. J Neurophysiol 107: 728–741, 2012. First published October 26, 2011; doi:10.1152/jn.00558.2011.—The development of central nervous system slice preparations for electrophysiological studies has led to an explosion of knowledge of neuronal properties in health and disease. Studies of spinal motoneurons in these preparations, however, have been largely limited to the early postnatal period, as adult motoneurons are vulnerable to the insults sustained by the preparation. We therefore sought to develop an adult spinal cord slice preparation that permits recording from lumbar motoneurons. To accomplish this, we empirically optimized the composition of solutions used during preparation in order to limit energy failure, reduce harmful ionic fluxes, mitigate oxidative stress, and prevent excitotoxic cell death. In addition to other additives, this involved the use of ethyl pyruvate, which serves as an effective nutrient and antioxidant. We also optimized and incorporated a host of previously published modifications used for other in vitro preparations, such as the use of polyethylene glycol. We provide an in-depth description of the preparation protocol and discuss the rationale underlying each modification. By using this protocol, we obtained stable whole cell patch-clamp recordings from identified fluorescent protein-labeled motoneurons in adult slices; here, we describe the firing properties of these adult motoneurons. We propose that this preparation will allow further studies of how motoneurons integrate activity to produce adult motor behaviors and how pathological processes such as amyotrophic lateral sclerosis affect these neurons.

green fluorescent protein; ethyl pyruvate; whole cell patch clamp; action potential; amyotrophic lateral sclerosis

Since the pioneering work of Henry McIlwain, brain slice preparations have become essential to investigate questions related to cellular neurophysiology (Collingridge 1995; Yamamoto and McIlwain 1966). These reduced preparations provide some advantages over in vivo preparations, in that responses are recorded in the absence of the influence of anesthetics while local functional synaptic circuitry may be maintained. More importantly, they permit excellent access to and recording from identified neuronal populations along with control of the external environment. This enables the superfusion of drugs and other agents at defined concentrations, which may be critical for the pharmacological/physiological characterization of the cell in question. Additionally, with regard to studies using intracellular electrodes, these preparations have an advantage in that they are more mechanically stable because of the lack of any cardiac or respiratory movements (Kerkut and Bagust 1995; Lipton 1986; Suter et al. 1999). On the other hand, slice preparations have certain disadvantages, including—particularly for studies of the spinal cord—the need to use tissue from neonatal animals because of the challenges of generating a preparation in which adult neurons are viable.

Spinal motoneurons (MNs), the “final common path” for all motor output from the spinal cord, have been studied in terms of motor physiology and have also been used as a classic model to study cellular neurophysiology (Brownstone 2006). Historically, recordings from spinal MNs have been obtained from either anesthetized or decerebrate cats with sharp microelectrode recordings (Eccles 1952; Woodbury and Patton 1952). With the advent of genetic tools, there has been a move to use mice as experimental models. In line with this, there have been some recent successful attempts to obtain microelectrode recordings from MNs in the adult rodent in vivo (Gardiner and Kernell 1990; Manuel et al. 2009; Meehan et al. 2010). However, recording from adult spinal MNs in vitro has remained a challenge. This is largely because of their sensitivity to ischemia, which is inevitable during the process of generating an in vitro preparation. This vulnerability may be related to the high metabolic demands and low calcium buffering ability of these cells (Duggal and Lach 2002; Nohda et al. 2007; Sakurai et al. 1997; von Lewinski and Keller 2005). It has also been observed that the large size of MNs—their dendrites may radiate up to 1 mm or more—makes them more susceptible to insults arising from transection of their processes during slicing procedures (Chen and Wolpaw 1994; Edwards and Konnerth 1992; Gibb and Edwards 1994; Luo et al. 2004; Moghaddasi et al. 2007).

Given these difficulties, investigators studying MNs have largely confined themselves to using spinal cord slices prepared from animals in the first 2 postnatal weeks (up to postnatal day P15) (Carlin et al. 2000; Thurbon et al. 1998). These young animals are more resistant to anoxia and have less myelination, which renders the tissue softer and more permeable to oxygen and thus more tolerant to insults imposed during the preparatory process (Gibb and Edwards 1994; Kerkut and Bagust 1995). However, with postnatal maturation there are clear changes in morphology as well as the constituent ion channels within MNs and other cells of the spinal cord. Therefore, while very useful, physiological data from younger age groups are not necessarily extrapolatable to older ages (Hori et al. 2001; Jiang et al. 1999; Li et al. 2005; Nakanishi and Whelan 2010; Song et al. 2006; Wilson et al. 2004). Furthermore, diseases affecting MNs such as amyotrophic lateral sclerosis (ALS) are adult-onset diseases. Even though...
some early changes in MN physiology have been seen in mouse models of ALS (Bories et al. 2007; Kuo et al. 2004; Quinlan et al. 2011), it will be necessary to study MNs at various postnatal stages in order to fully understand the physiological changes that occur during the disease.

A number of past attempts have been made to develop a viable adult in vitro spinal cord preparation for MN recording, with variable degrees of success (Fulton 1986; Hori et al. 2001; Jiang and Heckman 2006; Moghaddasi et al. 2007). A recent and successful attempt in this regard was by Carp et al. (2008) who, in the preparation of rat spinal cord slices, used polyethylene glycol (PEG) to limit the effects of the extensive membrane transections that occur during slicing and recorded intracellularly from presumed MNs in 6- to 12-wk-old rats with sharp microelectrodes. However, recordings from mouse lumbar MNs over P15 have rarely been reported, possibly because preparative and recording conditions have not been optimized. With adjustments to solutions used for rat recordings, some success has recently been reported in obtaining sharp electrode recordings from sacral MNs in an isolated mouse sacral spinal cord preparation (Jiang and Heckman 2006). This suggests that it should be possible to record, with patch-clamp techniques, mouse adult lumbar MNs in slice.

To determine the properties of defined populations of neurons, transgenic mice in which fluorescent proteins are expressed in genetically defined neurons have recently been used (Brownstone and Wilson 2008). To study the physiology of these delineated classes of cells, it becomes imperative that the investigator is able to identify and record from them under visual control. The whole cell patch-clamp technique has thus been the method of choice for such electrophysiological studies. Compared with sharp microelectrode recordings, the whole cell technique offers far better resolution, lower noise, and more robust determination of electrical parameters such as input resistance and membrane time constants (Li et al. 2004).

In this article, we describe a protocol for obtaining viable slices of the adult mouse lumbar spinal cord that permit whole cell recordings from MNs. While incorporating certain new modifications, our approach has largely been eclectic, incorporating a number of published neuroprotective manipulations used by other investigators in a variety of in vitro preparations. Largely this has meant that the approach relies on optimizing the design of the solutions used during the preparatory process to enhance their neuroprotective characteristics. We present an in-depth description of this empirically optimized preparative procedure, in addition to providing the rationale underlying the respective steps that constitute the process.

MATERIALS AND METHODS

Solutions. The artificial cerebrospinal fluid (aCSF) that was used for recording (RaCSF) was composed of (in mM) 121 NaCl, 3 KCl, 1.25 NaH2PO4, 25 NaHCO3, 1.1 MgCl2, 2.2 CaCl2, 15 dextrose, 1 (+)-sodium L-aspartate, 5 ethyl pyruvate, and 3 myo-inositol. This solution had a pH of ~7.4 (7.3 to 7.4 with moderate bubbling of 95% O2–5% CO2 (carbogen)) and an osmolality of 310 mosmol/kgH2O (range 305–315 mosmol/kgH2O). To ensure adequate oxygenation and appropriate pH adjustment, all bicarbonate-buffered solutions were bubbled with carbogen for at least 0.5 h prior to use and subsequently continuously throughout their usage. Both of the above solutions were made with distilled water, and the divalent ions were added last in order to avoid precipitation, only after bringing the volume close to the final and the pH approximately adjusted via bubbling with carbogen for at least 10 min. The DaCSF was chilled and kept on ice throughout. Both the DaCSF and RaCSF solutions were made fresh on the day of use. This is an aspect that makes the entire process more tedious, but we would like to emphasize that we have noticed a distinct decrease in the number and quality of labeled cells obtained when slices are prepared with solutions refrigerated overnight.

The intracellular pipette solution had the following composition (in mM): 131 K-methanesulfonate, 6 NaCl, 0.1 CaCl2, 1.1 EGTA-KOH, 10 HEPES, 0.3 MgCl2, 3 ATP-Mg2+ salt, 0.5 GTP-Na+ salt, 2.5 l-glutathione reduced, and 5 phosphocreatine di(tris) salt. The solution was adjusted to a pH of 7.25 with KOH (~10–15 mM of added K+ ions) and had an osmolality in the range of 295–300 mosmol/kgH2O. The solution was made with Milli-Q water (Millipore), divided into aliquots, and stored at −20°C. It was thawed, filtered through a 0.2-μm filter (Nalgene 4-mm cellulose acetate syringe filter), and kept on ice during use. This solution is designed largely to mimic the intracellular environment (Kay 1992) and, when used with the RaCSF and assuming complete exchange with the cellular contents, provides $E_K$ of ~98 mV, $E_C$ of ~75 mV (see Stil et al. 2009), $E_{Na}$ of ~79 mV, and a free Ca$^{2+}$ concentration in the range of 10 nM (Kay 1992). The solution has a junction potential of ~8 mV with the RaCSF (Jackson et al. 2004).

Especially when using mature tissue, it is critical that all extracellular solutions have a similar osmolality value, in order to avoid the exposure of the tissue to any osmotic shock (Moyer and Brown 1998). The intracellular solution was kept slightly hyposmolar to improve the stability of the recordings (Gibb 1995).

Animals and tissue preparation. All animal procedures were approved by the Dalhousie University Committee on Laboratory Animals and conform to the standards of the Canadian Council on Animal Care. Given that spinal MNs are cholinergic, transgenic mice in which enhanced green fluorescent protein (eGFP) was expressed under the control of the choline acetyltransferase (ChAT) promoter were used. These mice are based on a Swiss Webster background, express cytoplasmic eGFP, and were generated by the GENSAT Project at Rockefeller University (Gong et al. 2003).

The dissection was carried out in a Sylgard-lined aluminum dish that was placed on ice to cool. Animals beyond 6 wk of age (P42 and above) were considered as adult for the purposes of this study. Male or female adult mice were injected intraperitoneally with a mixture of ketamine (150 mg/kg body wt) and xylazine (20 mg/kg body wt). After losing their righting reflex, animals were placed in a supine position on a bed of ice until loss of the toe pinch response (~10–15 min). The slight hypothermia that was induced may serve to provide a certain degree of protection against subsequent insults (Gordon 2001). We have previously attempted transcardial perfusion of the animal with chilled DaCSF prior to decapitation but have not observed any substantial improvement in the quality of the slices obtained. It was thus decided to abandon its use in the interests of simplifying the protocol.

Usage. Both of the above preparations were then pinned down via the limbs onto the base of the cooled Sylgard-lined metal dish, which was filled with chilled DaCSF and kept on a bed of ice. The skin was removed, and the animal was eviscerated in order to expose the vertebral column. The DaCSF in the dish was exchanged for clean solution, and a vertebrectomy was performed to expose the spinal cord. The cord was held at the cervical end and lifted with a pair of forceps, and the dorsal and ventral roots were cut (relatively far

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...from the cord) with a pair of fine microscissors, so as to release the cord from the column. The isolated cord was gripped with forceps at the caudal end, and the roots were stripped by pulling them from the caudal toward the cervical end. This procedure not only strips the cord of its roots but also removes the overlying meninges. Although prima facie it may appear that this manner of mechanical stripping would be injurious to the MNs, and it may undeniably be so, we have persisted with it simply for the clean and complete removal of the meninges that it affords. In balance, we have found that an insufficient removal of the dura mater leads to a poorer cut through the cord, an aspect that has even more deleterious consequences for the health of the MNs. The lumbar enlargement of the cord was subsequently isolated and then transferred to a beaker containing clean 35°C prewarmed DaCSF. The beaker was kept at room temperature (RT), and its contents were bubbled with carbogen for 15 min. The underlying rationale was to give the cord some time to recover from the trauma of dissection and to allow it to equilibrate to a relatively higher temperature before being embedded into agarose.

Agarose-Low Melt (USB; no. 32830), a low-gelling-point agarose with a 1.5% gel point of 24–28°C, was used for purposes of embedding the cord. It is important to use an agarose with a sufficiently low gelling temperature in order to avoid exposing the cord to higher temperatures and a temperature shock with potentially deleterious consequences for the tissue. Empirically, we have found that the gel strength obtained by using a 4% (wt/vol) solution of the agarose in DaCSF was sufficient to permit a smooth cut through the adult spinal cord. Using a significantly lower gel strength leads to a poorer quality of slicing, because of compression of the cord by the blade, and in extreme cases causes the cord to be de-embedded out of the block, often with disastrous consequences for the tissue. The agarose was dissolved in DaCSF by heating in a microwave and then poured into a petri dish kept on ice. When the temperature at the center of the dish (point where cord is to be embedded) fell to around 29–30°C, the cord was drained of fluid by touching one end onto a Kimwipe moistened with DaCSF, inserted well below the surface into the still molten agarose, and straightened. All manipulations with the piece of the isolated lumbar cord were done by holding it at the upper lumbar end, since slices were generally accepted from the middle to lower lumbar region. The agarose with the cord was allowed to gel on ice, and ~5 min later when complete gelling had occurred, cold DaCSF was poured on top of the petri dish. While it might seem counterintuitive to wait 5 min in this environment, we found that this time was important in order to attain the gel strength necessary for optimal slicing. A block containing the embedded cord was cut out and fashioned by leaving approximately a couple of millimeters of agarose at the side edges and even less at the front edge that would meet the blade of the microtome. Approximately a centimeter length was retained at the back, to allow the blade to proceed beyond after slicing transversely through the cord. A 5% agar block was glued onto the cutting stage of the vibratome as a backing/support, and the agarose block containing the embedded piece of cord was glued in front (with the sacral end up). Ethyl cyanoacrylate glue (Loctite Super Glue Control, Henkel Canada) was used, and the stage was immediately immersed into chilled DaCSF to dissipate the heat that may be generated by the glue on curing. The cord was now ready to be sliced with a vibrating tissue slicer.

Our experience suggests that a smooth cut through the tissue is critical, as it helps maintain the relative integrity of the superficial layers of its roots but also removes the slice surface (50–70 μm), a region from which visually guided patch-clamp recordings are usually obtained. The blade should move through the tissue with relatively large-amplitude and high-frequency horizontal oscillations, coupled with a slow advance speed and relatively negligible vibrations in the vertical z-axis dimension (Geiger et al. 2002). We assert that with sensitive tissue such as the adult spinal cord the use of a tissue slicer with minimal z-axis vibration significantly enhances cellular viability. A vibrating tissue slicer (Vibratome 3000, Vibratome) was used to generate transverse slices of the spinal cord. One half of a double-edged carbon steel blade (Feather Blades; Canemco-Marivac, Lakefield, QC, Canada) was positioned at an angle of 20° to the horizontal plane, the vibratory amplitude setting was kept at 9.5 (the maximum setting is 10, which corresponds to an amplitude of 1.5 mm) with 60-Hz vibration frequency, z-axis peak-to-peak vibration was minimized to 3 μm, and the speed of advance was ~100 μm/s. [This was the slowest speed that our instrument would permit, although we speculate that dropping the speed further (~50 μm/s) may prove beneficial.] The slicing chamber was surrounded with ice, and the tissue was cut from the lateral edge of the cord. Invariably, the first few slices were discarded. We initially cut slices at a thickness of 450 μm to maximize dendritic arbor integrity but noticed that a better yield was obtained on reducing the thickness (325–350 μm), presumably because of better oxygenation and nutrient transfer to the slice core (Carp et al. 2008; Lipton 1986; Walz 1988). This also improved visualization of neurons in the slice.

After cutting, each slice was released from the agarose shell by blowing liquid at the edges of the slice and transferred with a fire-polished glass Pasteur pipette to 30% (wt/vol) PEG (Mn = 1,900–2,200; Aldrich no. 295906) in distilled water for 60 s, with fresh PEG solution used for each slice. The details of the PEG exposure protocol were as described by Carp et al. (2008). Each slice was then washed twice for a minute each with fresh 35°C DaCSF solution and then transferred to an incubation chamber containing 35°C DaCSF. The empty agarose shell was cut out, and further slices were obtained (~5–6 slices per preparation). After completion of slicing, all slices were allowed to incubate in 35°C DaCSF for a further 30 min, after which they were incubated in 35°C RaCSF for another half-hour. The chamber used for incubation was similar to the Gibb/Edward chamber, and slices within the chamber were kept suspended on a mesh and totally submerged under the solution (Edwards and Konnerth 1992; Gibb and Edwards 1994). Care was taken to avoid the clumping together of slices, and the quantity of solution used was kept at the minimum necessary to keep the slices completely submerged. The solution was continuously bubbled with carbogen, taking care to avoid undue mechanical vibration and trauma to the slices. Subsequently, the slices were transferred to RaCSF at RT and held under static interface conditions. Approximately 30 min was allowed to lapse prior to the slices being used for recording. Holding the slices under interface conditions clearly improved their viability compared with keeping them totally submerged. This is in agreement with previous observations that suggest that slices kept at the gas-liquid interface demonstrate better electrophysiological and morphological features compared with keeping them submerged (Aitken et al. 1995; Krimmer and Goldenk-Rakic 1997). The interface condition was roughly mimicked by lowering the fluid level to a point where only a thin film covered the upper layer of the slices, while their lower surfaces were in contact with the vigorously carbogenated incubation fluid. The assembly was kept closed by covering with a lid, thus creating a humidified atmosphere, and a tube was inserted to blow additional carbogen above the slices. While the postslicing incubation protocol ensured that the slices were heated at 35°C for 1–1.5 h, they were subsequently held at RT, since deterioration of slices is greater at higher temperatures (Lipton 1986). All recordings were performed at RT. In this manner, the slices remained healthy and could be used for at least 5–6 h beyond their preparation.

The above-described preparation protocol—from the outset when the animal is 10, its recordings will also occur when the first slice is ready for recording—was completed in 3 h.

**Whole cell patch-clamp recordings.** Once techniques were optimized such that there were fluorescent MNs in the slices, we proceeded with studying their viability with patch-clamp recordings. Electrophysiological recordings reported in this article were obtained from nine animals, although a larger number was used during procedure development and optimization. Individual slices were transferred to a polycarbonate recording chamber (model RC-22C; Warner In-
and the specific targeting of genetically identified neurons
Visual guidance ensures targeting of large ventral horn neurons
recordings of identified adult MNs could be reliably obtained.

RESULTS

Whole cell patch-clamp recordings were made from eGFP-labeled
MNs in the lateral ventral horn under visual guidance (Moyer and
Brown 2002; Stuart 2001). Invariably, the cells on the surface of the
slice were unhealthy or dead, and it was thus necessary to target cells
located relatively deeper. MNs that were either shrunken or lacked
shape and were swollen, had a spotty or extremely shiny appearance,
or lacked clear edges or whose internal organelles such as the
nucleus/nucleolus were visible were considered unhealthy and thus
rejected. We rarely encountered extremely unhealthy slices, which
were filled with craters of swollen and burst cells or for that matter
with apoptotic (shrunken and with membrane blebbing) cells, but often
obtained slices that lacked sufficient numbers of labeled cells (Liang
et al. 2007). The few that were present in such slices were often too
depth to be adequately visualized and thus unsuitable for visually
guided recordings. This situation was exacerbated at older ages and is
clearly the major challenge encountered during such recordings.
While permitting unequivocal identification of cell type, using the
presence of fluorescent labeling as an index for the assessment of cell
survival is in all likelihood a rather rigorous criterion, given that
during preparation the soluble protein may leak out of transected
processes, which in turn may eventually seal up and keep the cell
viable. In fact, in slices with a low yield of labeled cells, we often
observed a significant number of large neurons (>20-μm soma
diameter) in the area of the lateral ventral horn. By visual criteria these
cells were clearly healthy and were presumably MNs (Carlin et al.
2000; McHanwell and Biscoe 1981; Takahashi 1990; Thurbon et al.
1998).

Whole cell recordings were made with a MP-285 manipulator
(Sutter Instrument), a Multiclamp 700A patch-clamp amplifier (Mo-
olecular Devices), and a digitizer (Digidata 1322A; Molecular De-
VICES). Patch pipettes were fashioned from thin-walled borosilicate
glass tubing (BF150-110-10; Sutter Instrument) with a horizontal
puller (P-97 Flaming/Brown Micropipette Puller; Sutter Instrument),
were filled with intracellular pipette solution, and had resistances of
~2.5–3.5 MΩ. All current-clamp recordings were done in bridge
deep mode. Cells that were depolarized such that they spontaneously fired
action potentials on achieving whole cell mode were rejected from
further study. Data were acquired with the pCLAMP software suite
(pCLAMP 8.2; Molecular Devices). Analog signals were low-pass
filtered at 4 kHz and sampled at 20 kHz. All reported voltage values
were corrected post hoc for the liquid junction potential between the
intracellular pipette solution and RaCSF.

Data are expressed as means ± standard deviation (SD). Unless
otherwise mentioned, chemicals were obtained from Sigma-Aldrich
(St. Louis, MO).

Whole cell patch-clamp recordings and assessment of in-
trinsinc properties of adult lumbar MNs. To validate the prep-
paration, whole cell patch-clamp recordings were made from
eGFP-positive MNs in ChAT::eGFP mice. We had previously
established that eGFP-positive neurons in the ventral horn in
these mice are MNs, and that few if any of the other cholin-
ergic neurons express eGFP (Miles et al. 2007). All labeled
cells that we recorded were laterally positioned and located
ventral to the level of a putative horizontal axis passing through the
central canal, indicating that these cells were somatic rather than
autonomic MNs. The motoneuron shown in Fig. 1, C and
D, had a somatic long diameter of 30 μm. From a data set of
24 images of MN somas that were representative of the cells
that we recorded, we obtained an average soma long diameter of
35 ± 8 μm. A recent study reported that the mean diameter of
γ-MNs in P21 mice was 17 μm compared with 31 μm for
α-MNs (Friese et al. 2009). During recordings, we made an

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results

The goal in the development of this procedure was to
develop a preparation in which visually guided patch-clamp
recordings of identified adult MNs could be reliably obtained.
Visual guidance ensures targeting of large ventral horn neurons
and the specific targeting of genetically identified neurons
(e.g., with fluorescent protein expression in particular subpop-
ulations of MNs). The process of development of the prepara-
tion involved empirical optimization of the procedure and the
composition of the solutions. During the process of iteratively
optimizing the procedure, we evaluated each modification by
visual criteria, namely, the number of fluorescently labeled
MNs obtained and whether these cells appeared to be un-
healthy (e.g., if they were swollen or had a spotty appearance).
Figure 1, A and B, are representative low-magnification fluo-
rescence images obtained with a ×10 objective of one ventral
horn of the slice prepared from animals aged P43 and P48,
respectively. Surprisingly, with the slicing of the tissue by a
lateral approach, we commonly encountered significant label-
ing only within one ventral horn of the slice. Cutting in the
dorsoventral or ventrodorsal direction gave even lower yields.
We do not have an exact explanation for this observation, but
it may be related to the detrimental effects of rubbing of the
tissue with the surface of the blade. Figure 1, C and D, are
higher-magnification (×40) fluorescence and IR-DIC images,
respectively, from a representative slice prepared from a P45
animal. While the edges of the cell are extremely clear in the
fluorescence image (Fig. 1C), they are relatively harder to
discern in the IR-DIC image (Fig. 1D), presumably because
these cells are located below the surface of a relatively thick
slice. However, cells located close to the surface, while easier
to visualize and target, invariably do not provide satisfactory
recordings. It is thus a necessity that cells located well below
the surface be targeted for purposes of recording, since they
have a greater chance of providing a recording of superior
quality, as judged by criteria such as a robust resting membrane
potential (RMP) and the duration for which the recording may
be held. The individual cell shown in Fig. 1, C and D, is
probably located at depths that are close to the limits at which
we can record. Targeting cells deeper is akin to patching blind,
least under IR-DIC illumination. Fluorescence, on the other
hand, is not that steeply abated at these depths and may, if
necessary, be used as a guidance cue during the patching
process. However, even with such an approach, patching cells
that are very deep remains a challenge. Given these practical
limitations regarding which labeled cells may be recorded, it
follows that obtaining slices with an abundance of labeled cells
increases the probability of success. This is the preeminent
challenge when using animals of advanced ages. The animals
we usually recorded from were in the age range of 6–8 wk,
although the oldest that we have successfully recorded from is
P68.

Whole cell patch-clamp recordings and assessment of in-
trinsinc properties of adult lumbar MNs. To validate the prep-
paration, whole cell patch-clamp recordings were made from
eGFP-positive MNs in ChAT::eGFP mice. We had previously
established that eGFP-positive neurons in the ventral horn in
these mice are MNs, and that few if any of the other cholin-
ergic neurons express eGFP (Miles et al. 2007). All labeled
cells that we recorded were laterally positioned and located
ventral to the level of a putative horizontal axis passing through the
central canal, indicating that these cells were somatic rather than
autonomic MNs. The motoneuron shown in Fig. 1, C and
D, had a somatic long diameter of 30 μm. From a data set of
24 images of MN somas that were representative of the cells
that we recorded, we obtained an average soma long diameter of
35 ± 8 μm. A recent study reported that the mean diameter of
γ-MNs in P21 mice was 17 μm compared with 31 μm for
α-MNs (Friese et al. 2009). During recordings, we made an
attempt to target neurons with large soma sizes. This, coupled with their location and the fact that they are ChAT (eGFP) positive, made it likely that these cells were somatic lumbar H9251-MNs (Carlin et al. 2000; McHanwell and Biscoe 1981; Takahashi 1990; Thurbon et al. 1998). However, of the recorded cells, we only measured the somatic long diameters of two (cells 3 and 6 in Table 1 were 45 and 30/H9262 m, respectively), so we cannot be certain that all of the recorded neurons were H9251-MNs—some may have been H9253-MNs. After identification via fluorescence, cells were approached with a patch pipette under positive pressure and a gigaohm seal was obtained, after which the whole cell mode was achieved. Throughout the 1–2 h of recording, fluorescence of the patched neuron would typically diminish and often disappear because of dialysis of the eGFP protein, an effect similar to what we had reported for another cytoplasmic protein, ChAT (Carlin et al. 2000).

Figures 2 and 3 show example traces of recordings obtained from a single labeled adult MN (P47), which corresponds to cell 2 in Tables 1–3. They demonstrate that stable, visually guided whole cell recordings can be successfully obtained from these cells. The described preparatory procedure routinely yielded slices that were viable and visibly healthy. When the appropriate healthy cells were visually identified and targeted, we had close to a 100% success rate in obtaining whole cell recordings and in being able to hold the cells for close to 2 h. After the whole cell configuration was established, the cell was allowed to dialyze for 10 min to ensure reasonably complete exchange of the intracellular constituents. This is based on the fact that the exchange time constant is 3.4 min in spinal MNs (Palecek et al. 1999). Usually the RMP stabilized within that period. In case the RMP still showed signs of moving in a hyperpolarized direction, we waited for a further few minutes prior to recording data. Using such whole cell recordings, we have characterized the intrinsic passive and action potential firing properties, along with the properties of the individual action potentials of these adult lumbar MNs.

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>Age</th>
<th>IR, MΩ</th>
<th>RMP, mV</th>
<th>Rheobase, pA</th>
<th>Rheobase IR, mV</th>
<th>I-f Gain, Hz/pA</th>
<th>I-f Gain, Hz/mV</th>
<th>ARR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P54</td>
<td>166</td>
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<td>20</td>
<td>3.3</td>
<td>0.09</td>
<td>0.54</td>
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</tr>
<tr>
<td>2</td>
<td>P47</td>
<td>188</td>
<td>−74.2</td>
<td>25</td>
<td>4.7</td>
<td>0.1</td>
<td>0.53</td>
<td>1.67</td>
</tr>
<tr>
<td>3</td>
<td>P51</td>
<td>40</td>
<td>−68.9</td>
<td>295</td>
<td>11.8</td>
<td>0.021</td>
<td>0.53</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>P52</td>
<td>128</td>
<td>−74.8</td>
<td>70</td>
<td>9</td>
<td>0.086</td>
<td>0.67</td>
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</tr>
<tr>
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<td>P68</td>
<td>92</td>
<td>−73.5</td>
<td>90</td>
<td>8.3</td>
<td>0.115</td>
<td>1.25</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>P45</td>
<td>153</td>
<td>−73.3</td>
<td>20</td>
<td>3</td>
<td>0.121</td>
<td>0.79</td>
<td>1.83</td>
</tr>
<tr>
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<td>P50</td>
<td>180</td>
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<td>0.109</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>P43</td>
<td>49</td>
<td>−74.8</td>
<td>450</td>
<td>22</td>
<td>0.067</td>
<td>1.37</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>P50</td>
<td>110</td>
<td>−74</td>
<td>30</td>
<td>3.3</td>
<td>0.106</td>
<td>0.96</td>
<td>1.8</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>123 ± 54</td>
<td>−73 ± 2</td>
<td>113 ± 154</td>
<td>8 ± 6</td>
<td>0.09 ± 0.03</td>
<td>0.8 ± 0.3</td>
<td>1.8 ± 0.2</td>
</tr>
</tbody>
</table>

IR, input resistance; RMP, resting membrane potential; I-f gain, slope of the relationship between the frequency of spike firing vs. the injected stimulus current; ARR, anomalous rectification ratio.
We found that once the preparation was optimized and we began the electrophysiological experiments, recordings were obtained from nine consecutive preparations (100%). That is, from slices that were prepared with this protocol, we present data from nine adult MNs over P42 that were successfully recorded. Cells that spiked tonically in the absence of any bias current injection, and thus lacked a stable RMP, were rejected. The cells in this data set had an

Fig. 2. Depolarization-induced action potential firing of adult motoneurons. The example cell displayed is recorded from a mouse aged P47. A–D: voltage recordings showing a passive depolarization with smaller stimulus current (20 pA; D) and action potential firing with increasing frequency with higher stimulus current (40, 100, and 650 pA for C, B, and A respectively). Note the observance of some spike frequency adaptation in the initial part of A. Scaling is identical for A–D. E: plot of action potential frequency against the intensity of the stimulus current (I–F plot) for the cell shown in A–D. Solid line is a linear fit to the data. F: action potential elicited via a brief shock stimulus (10 ms) from the same cell. The intensity of the stimulus employed was of a magnitude required to just elicit an action potential (200 pA in this example cell). The trace displayed here is an average of 10 such sequentially obtained recordings. As indicated, the displayed spike waveform is truncated. The trace displays the fast afterhyperpolarization (fAHP) following the spike (indicated by arrow), which in turn followed by a prominent slow waveform of the medium afterhyperpolarization (mAHP; indicated by filled triangle) that follows an individual action potential.

We found that once the preparation was optimized and we began the electrophysiological experiments, recordings were obtained from nine consecutive preparations (100%). That is, from slices that were prepared with this protocol, we present data from nine adult MNs over P42 that were successfully recorded. Cells that spiked tonically in the absence of any bias current injection, and thus lacked a stable RMP, were rejected. The cells in this data set had an
average age of ∼P51 (Table 1) and a mean RMP of ∼73 ± 2 mV.

On stabilization of the RMP, the input resistance (IR) was measured by averaging ∼15 sweeps at 9-s intervals of 1.5-s-duration current steps that hyperpolarized the cell ∼3–5 mV below the RMP. The cells had an average IR of 123 ± 54 MΩ.

The cells were then depolarized sequentially with increasing positive current injections of 1-s duration approximately every 10 s. With increasing positive current injection, cells initially showed a passive ohmic response (Fig. 2D), after which they subsequently fired large overshooting action potentials. Rheobase was defined as the minimum magnitude of depolarizing current that was capable of eliciting 1 or 2 spikes from the RMP, elicited with a 1.5-s-long step. It should be noted that, prior to eliciting spiking, we did not equalize the RMP for each cell to a common value via injection of a bias current. The average rheobase value was 113 ± 154 pA. The product of the rheobase with the corresponding IR value suggested that, on average, these cells needed to be depolarized 8 ± 6 mV above the RMP to start spiking. With further depolarization, the frequency of firing increased with an increase in stimulation intensity (Fig. 2. A–C). Spike frequency was estimated by counting the number of action potentials within the 1-s-long step. A plot of the firing frequency versus the stimulus current (I-f plot) is shown in Fig. 2E. The average firing gain (I-f slope) was 0.09 ± 0.03 Hz/pA. Normalizing the firing gain to the IR led to a normalized gain expressed in units of hertz per millivolt (Baylor and Fettiplace 1979). From nine cells, we obtained a normalized firing gain of 0.8 ± 0.3 Hz/mV, thus suggesting that beyond the threshold voltage adult MN firing will increase at the rate of ∼4 Hz for current increments equivalent to generating 5 mV of passive depolarization. The I-f relation was linear (Fig. 2E) without any evidence of subprimary or secondary firing ranges.

Incremental hyperpolarizing current steps of 1-s duration were then injected at ∼10-s intervals (Fig. 3A). Figure 3 shows that small hyperpolarizations below the RMP elicit relatively passive responses, whereas larger hyperpolarizations elicit a time-variant depolarizing voltage sag. This feature is indicative of a time-dependent anomalous rectification corresponding to a decrease in slope resistance due to activation of the h conductance (Mayer and Westbrook 1983). This is illustrated in Fig. 3B, which is a voltage-current (V-I) plot of the data. Voltage values corresponding to the peak and steady-state voltages for each hyperpolarization have been plotted against the magnitude of the corresponding current step. The two values begin to deviate with larger hyperpolarizations, with the steady-state value eventually attaining a lower slope resistance, indicating anomalous rectification. Linear fits were made on the entire peak voltage plot and the latter linear part of the steady-state voltage plot for each cell to obtain the respective slope resistances. The degree of anomalous rectification was quantified by determining the anomalous rectification ratio (ARR), which was calculated by dividing the peak slope resistance by the steady-state slope resistance (Nelson and Frank 1967). We obtained an average ARR value of 1.8 ± 0.2. The above-described parameters are detailed in Table 1.

We have quantitatively characterized the parameters of individual spikes from a data set of seven cells with an average age of approximately P52 (Table 2). To do this in an objective manner, only the characteristics of the first spike elicited at rheobase were measured. For each cell, the values reported are averages obtained from five individual action potentials. Individual parameters (except action potential amplitude) were largely defined as in the study of Beurrier and colleagues on subthalamic nucleus neurons (Beurrier et al. 1999). The peak positive voltage of the spike overshoot is denoted as V0 and the peak negative voltage undershoot of the spike as Vu. The action potential voltage threshold (Vthr) was identified as the inflection point in the voltage trajectory preceding the upstroke—a point at which the spike is initiated and the membrane becomes strongly regenerative. The inflection point was determined by eye via inspection of either the raw voltage trace or its first time derivative. We obtained an average Vthr value of ∼57 ± 5 mV. Action potential amplitude, defined as (V0 – Vthr), averaged 73 ± 5 mV, and action potential width, measured as the time width at half the spike amplitude, had a mean value of 0.7 ± 0.1 ms. The maximum rate of rise during the upstroke of the action potential averaged 219 ± 33 mV/ms. This value is proportional to and thus indicative of the maximum net inward current (presumably dominated by the Na⁺ current) flowing across the membrane during the upstroke of the spike. Correspondingly, the maximum rate of fall during

Fig. 3. Hyperpolarization of adult lumbar motoneurons from rest. The example cell displayed is recorded from a mouse aged P47. A: voltage recordings showing a passive hyperpolarization with initial smaller stimulus current, followed by the appearance of a depolarizing voltage sag with larger hyperpolarizations. Displayed in the traces are 800 ms of recordings obtained with successive hyperpolarizing current increments of 40 pA. Open and filled circles denote the respective time points at which the peak and steady-state voltages were measured. B: voltage-current (V-I) plot of data from the cell shown in A. Peak and steady-state voltages of each trace have been plotted against the corresponding stimulus current. Symbols have the same meaning as in A.
the downstroke of the spike was $-121 \pm 27 \text{ mV/ms}$. Action potential time to peak was defined as the time difference between the spike initiation point ($V_{\text{thr}}$) and the point at which the peak of the action potential is reached ($V_o$). We obtained an average value of $0.7 \pm 0.1 \text{ ms}$ for this parameter. Action potential time to fall was defined as the time required for the spike voltage to fall from its peak down to a value equivalent to the $V_{\text{thr}}$. Spikes had an average time to fall value of $0.9 \pm 0.2 \text{ ms}$.

We next quantified the afterhyperpolarization (AHP). Spinal MNs have two components of the AHP (Mrowczynski et al. 2007; Nordstrom et al. 2007), a fast component (fAHP) lasting $\sim 2$–$10 \text{ ms}$ and a medium component (mAHP) lasting tens of milliseconds (Fig. 2F). The fAHP was quantified from the first spike elicited at rheobase and measured as the difference between the value of the peak hyperpolarizing voltage deflection immediately following the downstroke of the action potential ($V_u$) and the $V_{\text{thr}}$ for spike initiation. Adult MNs displayed an average fAHP amplitude of $15 \pm 5 \text{ mV}$. The apamin-sensitive mAHP is critical, as its duration determines the firing rate of mammalian MNs (Kernell 1984; Miles et al. 2007; Nordstrom et al. 2007). To study the mAHP, spikes were elicited with a brief shock stimulus of $10 \text{ ms}$ at $0.1 \text{ Hz}$. The amplitude of the shock was increased until it was just able to trigger an action potential. Ten such elicited voltage waveforms were averaged to characterize the mAHP. Figure 2F shows a spike (truncated in the figure) followed by a mAHP, which gradually decays to the RMP. We have quantitatively characterized the parameters of the mAHP (Mrowczynski et al. 2007) from a data set of five cells (Table 3) having an average age of $\sim P_{50}$. The mAHP amplitude was defined as the voltage difference between the negative peak of the mAHP and the RMP. Adult lumbar MNs had an average mAHP amplitude of $4 \pm 2 \text{ mV}$. The mAHP duration was taken as the time difference between the point at which the peak negative voltage undershoot of the spike was attained ($V_u$) and the point at which the mAHP waveform reached the baseline resting voltage ($R_{\text{MP}}$). We obtained a mean mAHP duration of $237 \pm 42 \text{ ms}$. mAHP time to peak was defined as the difference between two time points, $V_u$ and the peak negative voltage attained by the mAHP. We obtained an average value of $36 \pm 19 \text{ ms}$ for this parameter. The mAHP half-decay time was quantified as the time required for the mAHP waveform to decay from its peak voltage to a value corresponding to half the mAHP amplitude and averaged $57 \pm 10 \text{ ms}$.

While the above-mentioned parameter values are indicative of the properties of adult MNs, our main objective in performing these recordings was to validate the procedure and demonstrate that stable long-term recordings can be successfully obtained from adult MNs in these slices. A comprehensive study characterizing the complete postnatal maturation of MN intrinsic properties through adulthood has not been done. Comparisons with prior studies are fraught with difficulties introduced by species and strain differences and differences in techniques employed and solutions used. Nevertheless, we found some differences compared with previously published results.

The developmental maturation of the intrinsic properties of mouse lumbar MNs (up to P11) was recently studied with whole cell recordings in slices (Nakanishi and Whelan 2010). We report similar spike amplitude and voltage threshold values, but the spike width and action potential time to peak were lower in our recordings. These trends are fully consistent with and extrapolations of those observed in maturation until P8–P11. Compared with theirs, our data set seems to suggest that adult MNs have a significantly higher firing gain and smaller

### Table 2. Characterization of parameters of individual action potentials of adult motoneurons

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>Age</th>
<th>$V_o$, mV</th>
<th>$V_u$, mV</th>
<th>AP ampl, mV</th>
<th>AP Width, ms</th>
<th>$V_{\text{thr}}$, mV</th>
<th>TTP, ms</th>
<th>TTF, ms</th>
<th>fAHP ampl, mV</th>
<th>MRR, mV/ms</th>
<th>MRF, mV/ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P54</td>
<td>18.4</td>
<td>$-73.8$</td>
<td>80</td>
<td>0.78</td>
<td>$-61.4$</td>
<td>0.77</td>
<td>1</td>
<td>12.4</td>
<td>229.6</td>
<td>$-109.6$</td>
</tr>
<tr>
<td>2</td>
<td>P47</td>
<td>21.4</td>
<td>$-73$</td>
<td>75</td>
<td>0.59</td>
<td>$-53.8$</td>
<td>0.60</td>
<td>0.65</td>
<td>21.2</td>
<td>245.6</td>
<td>$-153.8$</td>
</tr>
<tr>
<td>3</td>
<td>P51</td>
<td>18</td>
<td>$-59.8$</td>
<td>65.6</td>
<td>0.75</td>
<td>$-47.6$</td>
<td>0.72</td>
<td>0.9</td>
<td>12.2</td>
<td>172</td>
<td>$-95.2$</td>
</tr>
<tr>
<td>4</td>
<td>P52</td>
<td>16</td>
<td>$-68$</td>
<td>71.6</td>
<td>0.81</td>
<td>$-55.6$</td>
<td>0.74</td>
<td>1</td>
<td>12.4</td>
<td>189</td>
<td>$-96$</td>
</tr>
<tr>
<td>5</td>
<td>P60</td>
<td>14.3</td>
<td>$-72.7$</td>
<td>72</td>
<td>0.74</td>
<td>$-57.6$</td>
<td>0.79</td>
<td>0.85</td>
<td>15.1</td>
<td>203.4</td>
<td>$-113.4$</td>
</tr>
<tr>
<td>6</td>
<td>P45</td>
<td>10.2</td>
<td>$-80$</td>
<td>68.6</td>
<td>0.49</td>
<td>$-58.4$</td>
<td>0.51</td>
<td>0.55</td>
<td>21.6</td>
<td>267.2</td>
<td>$-162.2$</td>
</tr>
<tr>
<td>7</td>
<td>P50</td>
<td>12.7</td>
<td>$-75.3$</td>
<td>77</td>
<td>0.73</td>
<td>$-64.3$</td>
<td>0.70</td>
<td>1</td>
<td>11</td>
<td>225.7</td>
<td>$-114$</td>
</tr>
</tbody>
</table>

Mean ± SD: $16 \pm 4$; $-72 \pm 7$; $73 \pm 5$; $0.7 \pm 0.1$; $-57 \pm 5$; $0.7 \pm 0.1$; $0.9 \pm 0.2$; $15 \pm 5$; $219 \pm 33$; $-121 \pm 27$.

Vo, peak voltage overshoot; Vu, peak voltage undershoot; AP ampl, action potential amplitude; AP width, action potential width at half-amplitude; $V_{\text{thr}}$, action potential threshold voltage; TTP, time to peak of the action potential; TTF, time to fall for the action potential; fAHP ampl, amplitude of the fast afterhyperpolarization; MRR, maximum rate of rise during the upstroke of the action potential; MRF, maximum rate of fall during the downstroke of the action potential.

### Table 3. Characterization of parameters of the medium afterhyperpolarization

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>Age</th>
<th>mAHP TTP, ms</th>
<th>mAHP Duration, ms</th>
<th>mAHP HDT, ms</th>
<th>mAHP ampl, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P54</td>
<td>36.2</td>
<td>223</td>
<td>72.2</td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td>P47</td>
<td>34.8</td>
<td>288.4</td>
<td>58.7</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>P51</td>
<td>64.6</td>
<td>271.7</td>
<td>55</td>
<td>1.8</td>
</tr>
<tr>
<td>4</td>
<td>P52</td>
<td>28.2</td>
<td>184.3</td>
<td>52.3</td>
<td>3.97</td>
</tr>
<tr>
<td>5</td>
<td>P45</td>
<td>13.8</td>
<td>219.8</td>
<td>46.02</td>
<td>7.25</td>
</tr>
</tbody>
</table>

Mean ± SD: $36 \pm 19$; $237 \pm 42$; $57 \pm 10$; $4 \pm 2$.

mAHP, medium after hyperpolarization; mAHP TTP, time to peak of the mAHP; mAHP duration, total duration of the mAHP; mAHP HDT, half-decay time of the mAHP; mAHP ampl, amplitude of the mAHP.
Innovative Methodology

rheobase value, both suggesting an enhanced excitability compared with P8–P11 MNs (Nakanishi and Whelan 2010).

Comparison with in vivo recordings from adult MNs in the mouse is definitely complicated, since both the published studies use intracellular rather than patch recording techniques (Manuel et al. 2009; Meehan et al. 2010). Thus it comes as no surprise that the absolute values of input resistance and firing gain are about an order of magnitude lower, and rheobase an order of magnitude higher, than those obtained by us or in the study by Nakanishi and Whelan (2010). The ARR values obtained by us and Manuel et al. (2009) are, however, comparable. We also note that the in vivo studies report mAHP parameters such as duration and half-decay time that are significantly shorter than those reported either by us in the present study or by Nakanishi and Whelan (2010) for P8–P11 MNs. Thus it can be seen that a more extensive study would be necessary to understand changes in MN properties through development and aging. The techniques presented here will allow for such comprehensive investigations.

DISCUSSION

In this article, we describe an adult mouse in vitro spinal cord slice preparation in which whole cell patch-clamp recordings from lumbar MNs can be obtained. Along with the preparatory procedure, we present electrophysiological recordings from adult mouse MNs and quantify the intrinsic spiking properties of these cells. These recordings are presented primarily with the intention of validating the procedure, rather than with the objective of a comparative characterization with previously published studies.

In developing such a preparation, it is inevitable that optimization is to some extent empirical and subjective. For instance, as stated above, we invariably gauged the suitability of a parameter change by assessing the number of fluorescent cells obtained and also made a subjective assessment of their appearance. However, similar to investigators who have optimized their respective in vitro preparations from different central nervous system regions, we had underlying rationales for the parameter changes. Here we discuss the rationales as related to the development of this preparation. Our primary approach was based on improving the neuroprotective ability of the solutions that were used. Accordingly, we not only have bolstered the DaCSF but also have supplemented the RaCSF that was used for recording and storage of the slices. It should be noted that this is in contrast to the commonly used minimal RaCSF composition of inorganic salts and glucose. Nevertheless, we believe that this is a valid approach, since our objective was to use a solution that not only provides physiological ion concentrations but also secures maximal cellular viability while permitting the recording of robust baseline physiological parameters (e.g., spike amplitude, RMP) comparable to those expected in vivo.

Limiting energy failure. The key challenge in developing this technique was to mitigate energy failure that results from the disruption of blood circulation during preparation (Lipton 1999). Glucose is the main energy source in the mammalian brain and is metabolized either oxidatively or nonoxidatively (glycolysis). Hypoxia contributes to an abrogation of oxidative phosphorylation, which generates most ATP. Energy needs must now be met solely by glycolysis, a process that in normal circumstances supplies only 5% of a neuron’s energy needs (Silver and Erecinska 1998). Under resting conditions, nearly 50–60% of the ATP produced in the brain is used to support and maintain the ionic disequilibrium across the plasma membrane, without which no electrical signaling can occur (Silver and Erecinska 1998). A fall in the ATP levels results in failure of the ATP-dependent Na⁺-K⁺ pump, and thus accumulation of Na⁺ ions intracellularly. Na⁺ ions in turn pull Cl⁻ ions through the passive Cl⁻ leak, which results in intracellular accumulation of NaCl, an increase in osmotically driven entry of water into the cell, and thus cellular swelling (Hansen 1985; Rothman 1985). This process, known as cytotoxic edema, results in eventual death in a process known as oncosis (Liang et al. 2007; Won et al. 2002). Performing the bulk of the preparative procedure under cold conditions is one attempt to lower the metabolic rate and thus maintain better tissue ATP/energy levels to limit oncosis (Erecinska et al. 2003).

In addition, we ensured that there was an appropriate glucose supply. Normal glucose levels in the cerebrospinal fluid range between 0.5 and 5 mM. However, a survey of brain slice preparation protocols would show that hyperglycemic concentrations (range of 10–25 mM) are typically used (An et al. 2008; Markram et al. 1997; Moyer and Brown 2002; Shi and Liu 2006). This is considered essential, as in the absence of a blood supply nutrients must diffuse into the deeper layers of the tissue from the extracellular fluid. For adult MNs, we have empirically determined optimal dextrose levels to be in the range of 15–20 mM.

We also added ethyl pyruvate, an aliphatic ester of the monocarboxylic acid pyruvic acid. Pyruvate is an endogenous metabolite of glycolysis and either enters the mitochondria for subsequent oxidation in the tricarboxylic acid (TCA) cycle or is anerobically converted to lactate in the cytosol. Thus pyruvate, when added exogenously, should bypass glycolysis and enhance the energy status of the cell by promoting ATP generation via the TCA cycle (Hertz and Diener 2002). Under conditions in which oxygen is lacking, pyruvate is converted to lactate (which subsequently passes to the extracellular space and is washed away) by cytosolic lactate dehydrogenase. This generates NAD⁺ and increases the cytosolic NAD⁺/NADH ratio, which in turn is critical for preventing glycolytic arrest. Thus, under anerobic conditions, pyruvate helps to generate ATP by sustaining anerobic glycolysis (Hertz and Diener 2002; Park et al. 1998; Taylor et al. 2005).

Given these considerations, a number of previously published brain slice preparation protocols have supplemented their solutions with pyruvic acid added as a sodium salt (Forsythe 1994). However, sodium pyruvate is unstable and dimerizes rapidly in aqueous solutions (~20 min) into the metabolically inactive pyruvate hydrate and subsequently more slowly into parapyruvate—a compound that acts as an inhibitor of the TCA cycle and would thus be detrimental to the already compromised energetics of the cells in vitro (Montgomery and Webb 1956; Willems et al. 1978; Zeng et al. 2007). Thus it would appear that supplementing solutions with inorganic salts of pyruvic acid would, at minimum, prevent a full realization of the potential benefits of exogenously added pyruvate, either as an energy source or as a mitigator of oxidative stress (described below).

These problems may be overcome by use of the ethyl ester of pyruvic acid, ethyl pyruvate (Sims et al. 2001), which serves...
as a prodrug of pyruvate and is relatively more lipophilic. It thus penetrates into cells, where it is subsequently hydrolyzed by intracellular esterases to release pyruvate. This is in contrast to the use of an inorganic pyruvate salt, whereby uptake of pyruvate would be mediated by proton-linked monocarboxylate transporters, whose integrity may potentially be compromised by the prevalent conditions of oxidative stress (Aghajanian and Rasmussen 1989). Moreover, deesterification establishes a transcellular gradient that in turn favors further entry of the ethyl ester, thus allowing high levels of pyruvate to accumulate intracellularly (Malaisse et al. 1996; Mertz et al. 1996; Sims et al. 2001). Ethyl pyruvate may even traverse the intracellular membranes and accumulate in significant levels within mitochondria, which is the primary site for oxidative metabolism, thus augmenting its neuroprotective effects. In addition to functioning as a metabolic substrate, pyruvate also serves as an endogenous scavenger of prooxidant moieties and would thus be neuroprotective in its actions (Dobsak et al. 1999; Kim et al. 2005; O’Donnell-Tormey et al. 1987; Sims et al. 2001). Thus, compared with exogenously added inorganic pyruvate salts, the use of ethyl pyruvate permits a far more efficient antioxidant protection and metabolic boost (Fink 2008; Mertz et al. 1996; Zeng et al. 2007). It has been shown to have neuroprotective effects in the postischemic brain and in a mouse model of spinal cord injury (Genovese et al. 2009; Kim et al. 2005; Yu et al. 2005). We have supplemented our aCSF solutions with ethyl pyruvate at a concentration of 5 mM (Varma et al. 1998; Wang et al. 2005). Addition of higher concentrations (10 mM) did not prove beneficial and, as judged from the appearance of the cells within the slice, may even have had some deleterious effects, perhaps through its conversion to lactic acid and the resultant acidosis (Desagher et al. 1997; Sims et al. 2001; Yi et al. 2007) or through the effects of the intracellularly released ethanol (Videla and Valenzuela 1982). We also experimented with malate, another TCA cycle intermediate, in order to further the neuroprotective potential of the solutions via an enhanced preservation of mitochondrial function. However, solutions supplemented with diethyl malate (1 mM) in addition to ethyl pyruvate did not demonstrate any marked improvement (Gramsbergen et al. 2000; Herrington et al. 1996; Hertz and Diener 2002).

Substitution of ions and use of an osmolyte in extracellular solutions. Given the effects of intracellular Na⁺ accumulation, one of the earliest approaches used to generate viable slices to record MNS involved substitution of the Na⁺ ions (NaCl) in the aCSF with an impermeant molecule such as sucrose (Aghajanian and Rasmussen 1989). Investigators over the years have tried other Na⁺ substitutes instead of sucrose with variable benefits (Tanaka et al. 2008). In light of a published report (Ye et al. 2006), we initially attempted to substitute the Na⁺ ions with glyceral but obtained very little benefit and thus reverted back to using sucrose as a Na⁺ substitute in the DaCSF. Moreover, we found that substitution of the 26 mM Na⁺ in the NaHCO₃ buffer through use of a choline bicarbonate buffer in the DaCSF resulted in an improvement in viability (Richerson and Messer 1995). Also, substitution of NaCl results in removal of Cl⁻ anions, without which no oncosis would occur (Olney et al. 1986; Rothman 1985). We have further lowered the Cl⁻ concentrations in the DaCSF by the use of alternative salts such as K-gluconate instead of KCl and MgSO₄ instead of MgCl₂. The residual Na⁺ and Cl⁻ concentrations in the DaCSF are thus minimal (from the added 1 mM Na-ascorbate, 2 mM Na-kynureinate, and 1 mM CaCl₂).

The hypoxia-induced failure of the Na⁺-K⁺-ATPase pump and the dissipative loss of K⁺ ions from inside the cell result in anoxic depolarization, which in turn may lead to opening of voltage-dependent channels, including Ca²⁺ channels, thus promoting accumulation of Ca²⁺ ions within the cell. Accumulation of intracellular Na⁺ ions, coupled with the depolarization, also promotes a reverse operation of the Na⁺/Ca²⁺ exchanger. This, coupled with the failure of the Ca²⁺-ATPases, leads to Ca²⁺ loading of the cells and the associated cytosolic effects of high intracellular levels of Ca²⁺ ions (Hansen 1985; Heath and Shaw 2002; Stys 2004; Won et al. 2002). Thus it would appear that blocking Ca²⁺ influx pathways would have a salutary effect on the neurons. It may intuitively appear that eliminating all extracellular Ca²⁺ in the DaCSF along with raising the Mg²⁺ levels to block voltage-gated Ca²⁺ channels should enhance the degree of neuroprotection. However, in doing so, we have found the slices to be unhealthy. While attempting to record, we observed the surface of these slices to be rigid and difficult to break through. This may have resulted in greater contamination of the glass with tissue debris, resulting in far lower success rates in obtaining gigahm seals. Even when seals were obtained and a successful break-in achieved, we observed that it was hard to maintain these recordings beyond a few minutes. One putative explanation of such poor cell quality may be that neurons presumably need a “Ca²⁺ set point”—an optimal range of intracellular Ca²⁺ concentration—in order to survive. Too high concentrations cause the cell to die by necrosis, whereas too low a concentration is also lethal via promoting apoptosis (Aitken et al. 1995; Zipfel et al. 2000). Moreover, there is also the possibility that neurons are susceptible to injury via the “Ca²⁺ paradox” effect, whereby restoration of normal Ca²⁺ levels after exposure to a Ca²⁺-free environment leads to a paradoxical marked increase in intracellular Ca²⁺ concentration (Kosugi et al. 2008). On further experimentation, we empirically ascertained that a Mg²⁺-to-Ca²⁺ ratio of 4:1 in the DaCSF permitted cell survival while avoiding the above-mentioned problems (Moyer and Brown 1998).

Another additive in our aCSF solutions is the polyhydric alcohol myo-inositol (3 mM) (Forsythe 1994; Klug and Trussell 2006). Myo-Inositol, along with being involved in the generation of intracellular signaling compounds such as inositol 1,4,5-trisphosphate (IP₃), is a major organic osmolyte in cells (Cordoba et al. 1996; Fisher et al. 2002). Organic osmolytes are molecules that are accumulated within cells and are secreted to adaptively counter the influx of water and subsequent changes in cell volume. Their relatively inert nature allows cells to tolerate significant changes in their intracellular concentrations without any major effects on cellular structure or function. As discussed above, cells within the slice have a tendency to swell, and this continues into the postpreparatory period (Brahma et al. 2000; Siklos et al. 1997). The depletion of myo-inositol from cells that occurs during the preparative phase would compromise the ability of cells to counter the osmotically mediated water influx and also to maintain sufficient levels of the inositol phosphate signaling molecules (Fisher et al. 2002). We therefore supplemented the aCSF solutions with myo-inositol, with the intention that the neurons would take up the molecule and thus replenish its loss. We did
observe a distinct benefit, in that it was now easier to move into the whole cell configuration. We do not know an exact explanation for this, other than to generally surmise that it probably reflects greater membrane integrity arising from enhanced cellular viability.

Limiting excitotoxicity and the use of antioxidants. Increases in extracellular glutamate can lead to neuronal death through excitotoxic processes (Lau and Tymiński 2010). Presynaptic loading with Na$^+$ ions, coupled with depolarization, leads to a reversal of the Na$^+$-dependent glutamate transporter, which may cause glutamate release and a resultant buildup of glutamate in the synaptic cleft (Grewer et al. 2008; Stys 2004; Szatkowski et al. 1990). This would activate AMPA/kainate and NMDA receptors, leading to influx of Na$^+$ and Ca$^{2+}$ ions. MNs are especially sensitive to this excitotoxicity, since they contain Ca$^{2+}$-permeable AMPA receptors (Van Den Bosch et al. 2006; von Lewinski and Keller 2005). Furthermore, the paucity of Ca$^{2+}$-binding proteins in spinal MNs results in low cytosolic Ca$^{2+}$ buffering capacity and thus a high vulnerability to any increase in intracellular Ca$^{2+}$ concentration (von Lewinski and Keller 2005). In addition, a rise in cytosolic levels of Ca$^{2+}$ ion along with its consequent uptake into mitochondria results in inhibition of oxidative phosphorylation and enhanced generation of reactive oxygen species (ROS) including H$_2$O$_2$, superoxide, and peroxynitrite, thus promoting oxidative stress (Won et al. 2002). These ROS can rapidly react with DNA or cause lipid peroxidation and membrane damage, in addition to irreversibly damaging critical proteins, often with severe consequences for the cell (Halliwell 1992; Raha and Robinson 2000; Reynolds et al. 2007). Spinal MNs are far more vulnerable to the consequences of mitochondrial dysfunction compared with other cell types, such as dorsal horn neurons (Kaal et al. 2000). We therefore used techniques to limit excitotoxicity and the effects of oxidative stress.

The use of glutamate antagonists should thus afford an enhanced degree of neuroprotection. We have confirmed that addition of the glutamate antagonist kynurenate acid at concentrations that block NMDA and non-NMDA receptors (2 mM) in the DaCSF had a salutary effect on cell viability. This concentration presumably also blocks α7 nicotinic acetylcholine receptors, which may be important given the presence of choline, a nicotinic agonist, in our DaCSF (Alkondon et al. 1997; Hilmas et al. 2001; Phillis et al. 1999). In addition, the use of ketamine in the anesthetic cocktail may have additionally contributed to glutamate receptor blockade, since it is known to antagonize NMDA receptors (Chizh 2007).

We attempted to lower the levels of oxidative stress with the antioxidant sodium L-ascorbate (vitamin C) in both the DaCSF and RaCSF solutions to scavenge cytotoxic free radicals. (Ascorbate may additionally exert neuroprotective effects by promoting the uptake of glutamate via a glutamate/ascorbate exchange.) Ascorbate and reduced glutathione (GSH) are the two major water-soluble antioxidants in the brain that are diminished after anoxic depolarization during slice preparation. Ascorbate has an average neuronal intracellular concentration of ~10 mM, and the L-stereoisomer can be taken up from the extracellular fluid by neurons. GSH, on the other hand, is not taken up by cells from the bathing solution and was thus included in the intracellular pipette solution at its endogenous neuronal concentration of 2.5 mM (Rice 1999; Rice et al. 1994). A survey of various slice preparation protocols revealed that ascorbate is generally supplemented in the range of 0.4–1 mM, with 0.4 mM being the endogenous concentration in the extracellular fluid (Moyer and Brown 2002; Rice 2000). We have used a concentration at the higher end of the spectrum (1 mM). An interesting aside is that brain ascorbate levels are extremely high in turtles, and may be responsible for the robustness of in vitro turtle spinal cord preparations (Lutz and Milton 2004; Rice 1999, 2000). Both ascorbate and its oxidized analog dehydroascorbate (subsequently reduced intracellularly to ascorbate) are taken up by cells via transporter-mediated mechanisms (Rice 2000). To bypass such a step and with the expectation of achieving a higher intracellular antioxidant concentration, we experimented with substitution of ascorbate in our solutions with TEMPOL (0.75 mM), a synthetic membrane-permeant superoxide dismutase mimetic (Tal 1996). However, we did not observe any additional improvement and have thus retained the endogenously occurring ascorbate in our solutions.

Use of polyethylene glycol and subsequent incubation at higher temperatures. Immediately after slicing, slices were exposed to an aqueous solution of PEG. PEG was first demonstrated to enhance neuronal survival of an in vitro preparation by Carp et al. (2008). We have confirmed that PEG treatment enhances neuronal viability and have incorporated their procedure into our protocol. Presumably, PEG works as a membrane fusogen and limits the mechanical insult through promoting a rapid resealing of membrane breaches, thus enhancing neuronal survival. Previous studies have suggested that PEG, via removal of water from hydrophilic groups on the membrane surface, brings about a fusion of closely abutted cell membranes. In addition, it has been observed that PEG treatment reduces the level of oxidative stress. This may be a consequence of membrane refusion and thus lower Ca$^{2+}$ entry, although it has been suggested that PEG, by entering the cell and directly interacting with intracellular organelles as mitochondria, furthers their membrane integrity and thus their functioning (Luo et al. 2004; Luo and Shi 2007; Shi et al. 1999). We also speculate whether the short exposure (1 min) of the slices to an aqueous solution of PEG, which was not bubbled with gas and had no added nutrient source, conferred a degree of neuroprotection via the phenomenon of ischemic-hypoxic preconditioning (Samoilov et al. 2003).

While PEG undoubtedly promotes membrane resealing, it is likely that not all neuronal processes (especially those with residual smaller membrane breaches) are completely resealed by this treatment (Luo et al. 2004). Membrane resealing occurs at temperatures beyond 31°C, with significant resealing being obtained within 60 min (Shi and Pryor 2000). We therefore attempted to reseal residual membrane breaches by transferring the slices to 35°C DaCSF after their initial preparation at low temperatures and subsequent PEG exposure (Carp et al. 2008; Hestrin and Armstrong 1996; Markram et al. 1997). Given that at this point the slices still retain membrane breaches, we reasoned it would be prudent to do the initial heating in DaCSF, a solution designed to have minimal amounts of Na$^+$ and lower levels of Ca$^{2+}$ ions. They were subsequently incubated for a further 30 min in 35°C RaCSF, which contains physiological ion concentrations. We have empirically confirmed that the post slicing heating does have a beneficial effect on cell viability compared with incubating them at RT.
Summary. In this article, we have described a protocol for generating a mouse in vitro spinal cord slice preparation that allows the study of identified adult lumbar MNs using whole cell patch-clamp techniques. Furthermore, since MNs as a class are selectively vulnerable, a preparation optimized for in vitro MN recordings should prove robust in the study of other spinal neurons. While we have developed an empirically optimized procedure, the scope for other parametric combinations potentially leading to further improvement surely remains. With the advent of genetic approaches, use of the mouse as a model in spinal cord research is becoming increasingly common (Brownstone and Wilson 2008). Transgenic approaches offer a wide array of advantages, and we believe that this preparation will prove to be a valuable tool in studies aimed at understanding postnatal development of MN properties and spinal motor networks and of mouse models of diseases. Specifically, this preparation would be useful for pharmacological study and characterization of intrinsic properties of MNs at adult ages in mouse models of adult-onset neurodegenerative conditions such as ALS.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.M. and R.M.B. conception and design of research; P.M. performed experiments; P.M. analyzed data; P.M. and R.M.B. interpreted results of experiments; P.M. prepared figures; P.M. drafted manuscript; P.M. approved final version of manuscript.

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