Postnatal changes in the inactivation properties of voltage-gated sodium channels contribute to the mature firing pattern of spinal motoneurons

K.P. Carlin¹*, J. Liu¹, L.M. Jordan¹

¹Department of Physiology
University of Manitoba
Winnipeg, Manitoba R3E 0W3
Canada

* Current address
Purdue Pharma LP.
Discovery Research
Cranbury, NJ 08512
USA

Corresponding author:
Larry M. Jordan, Ph.D.
Professor, Department of Physiology
Spinal Cord Research Centre
The University of Manitoba
730 William Avenue, BMSB 425
Winnipeg, Manitoba R3E 3J7 CANADA
Tel: (204) 789-3534, (204) 789-3209 (lab)
Fax: (204) 789-3930
Email: larry@scrc.umanitoba.ca

Abbreviated title: Functional changes in motoneuronal sodium currents
ABSTRACT

Most mammals are born with the necessary spinal circuitry to produce a locomotor-like pattern of neural activity. However, rodents seldom demonstrate weight-supported locomotor behavior until the second or third postnatal week, possibly due to the inability of the neuromuscular system to produce sufficient force during this early postnatal period. As spinal motoneurons mature they are seen to fire an increasing number of action potentials at an increasing rate, which is a necessary component of greater force production. The mechanisms responsible for this enhanced ability of motoneurons are not completely defined. In the present study we assessed the biophysical properties of the developing voltage-gated sodium current to determine their role in the maturing firing pattern. Using dissociated postnatal lumbar motoneurons in short-term culture (18 – 24 hrs) we demonstrated that currents recorded from the most mature postnatal age group (P10 – 12) were significantly better able to maintain channels in an available state during repetitive stimulation than the younger age groups (P1-3, P4-6, P7-9). This ability was correlated with the ability of channels to recover quicker and more completely from an inactivated state. These age-related differences were seen in the absence of changes in the voltage-dependence of channel gating. Differences in both closed-state inactivation and slow inactivation were also noted between the age groups. The results indicate that changes in the inactivation properties of voltage-gated sodium channels are important for the development of a mature firing pattern in spinal motoneurons.
INTRODUCTION

The circuitry necessary to produce a locomotor pattern of muscle activation is already present and functional in the embryonic spinal cord of most mammals (Ren and Greer, 2003; Whelan, 2003). For example, rodents are capable of swimming and crawling at birth, however, the ability to raise the body off the ground and walk does not develop fully until the second or third postnatal weeks (Westerga and Gramsbergen, 1990; Brocard et al. 1999; Jiang et al. 1999a). One important feature of postnatal neuromuscular development is the matching of motoneuron discharge rates with the twitch properties of the muscles they innervate (Vrbova et al. 1985). This matching likely ensures the force generation in limb muscles necessary for the production of over-ground locomotion (Martin-Caraballo et al. 2000). As the muscle twitch time decreases during postnatal development (Close, 1964) a corresponding increase in the motoneuronal firing rate is required.

During the late embryonic and early postnatal periods, many types of motoneurons demonstrate an increase in maximal firing rate and duration of repetitive firing. This developmental phenomenon has been described in phrenic (Martin-Caraballo and Greer, 1999; Greer and Funk, 2005), oculomotor (Carrascal et al. 2006), hypoglossal (Viana et al. 1995) and spinal motoneurons (Vrbova et al. 1985; Fulton and Walton, 1986; Vinay et al. 2000). This ability of motoneurons to discharge action potentials at an increased rate has been attributed to various factors including an increase in sodium current density (McCobb et al. 1990; Garcia et al. 1998; Gao and Ziskind-Conhaim, 1998), development of repolarizing conductances (McCobb et al. 1990; Viana et al. 1994; Vinay et al. 2000), AHP amplitude increase (Fulton and Walton, 1986), the development of calcium channels (McCobb et al. 1989; Mynlieff and Beam, 1992; Miles et al. 2004) or changes in modulatory inputs (reviewed in Kernell 2003; Schmidt and Jordan, 2000). In addition to an increase in firing frequency, a second defining characteristic of mature spinal motoneurons is their ability to generate plateau potentials (Perrier and Hounsgaard, 2000). When the conductances underlying the plateau are activated, they create a second quasi-stable potential approximately 10 - 20 mV more positive to the resting potential (Hounsgaard et al. 1986; Hounsgaard and Kiehn, 1989).
Depending on the class of motoneuron, this underlying depolarization can persist for many seconds (Lee and Heckman, 1998). In order for a cell to fire a train of action potentials at such depolarized membrane voltages, sodium channels must resist entering an inactivated state so as to remain available for activation. In the present study we assess the biophysical properties of the sodium current generating the action potential as a potential mechanism contributing to postnatal development of the firing properties of spinal motoneurons.

In the mammalian nervous system, voltage-gated sodium currents can be generated by nine different channel subtypes (Catterall et al. 2003), each displaying some unique biophysical properties (Rush et al. 2007). The sodium channel subtype expression in mammalian spinal cord changes during the early postnatal period as demonstrated by studies examining expression at either the protein (Gordon et al. 1987; Schaller and Caldwell, 2000) or mRNA level (Beckh et al. 1989; Felts et al. 1997; Garcia et al. 1998; Schaller and Caldwell, 2000). Some of these changes have been directly localized to the spinal motoneurons (Schaller and Caldwell, 2000) and have been supported by electrophysiological data from mice expressing a spontaneous mutation in a late-developing sodium channel subtype (med mutant; Garcia et al. 1998). Taken together, these data suggest that a change in the biophysical characteristics of the sodium channels may, in part, be responsible for the change in firing properties of developing spinal motoneurons. But this type of kinetic data is absent from the literature on motoneurons, in all likelihood due to the inherent difficulties associated with voltage- and space-clamping the sodium current in these cells (e.g., Garcia et al. 1998).

In the present study we circumvent these technical difficulties by using dissociated postnatal lumbar spinal motoneurons cultured overnight in conditions that did not promote process formation. We then tested the hypothesis that the ensemble sodium current demonstrates age-related biophysical changes consistent with the known mature firing pattern of these cells. Specifically, we assessed the ability of the maturing complement of sodium channels to resist accumulating in an inactivated state during repetitive depolarizing stimulations that mimicked repetitive firing and evaluated the tendency of these channels to undergo closed-state inactivation during prolonged
depolarizations similar to the voltage change sensed by these channels during the expression of a plateau potential.
METHODS

Cell culture

Mixed spinal cord cultures were prepared from the lumbar spinal cords of BALB/c mice (P1 – P12; where P0 is the day of birth). Motoneurons were identified in the cultures by pre-labeling these cells through an intraperitoneal injection of the fluorescent maker Fluoro-Gold (10-20 µl of a 2% solution; Fluorochrome, Denver, CO) at least 24 hours prior to sacrifice (Miles et al. 2005). Spinal cords were isolated and transverse slices prepared from the lumbar cord (~L1 to L5 segments) as described previously (Carlin et al. 2000a). Care was taken to ensure that there were no adherent dorsal root ganglia. This resulted in spinal cord slices in which labeling was restricted to ventral motoneurons (Fig. 1A). After removing the agar from the periphery of the slices, the slices were placed in a calcium- and magnesium-free room temperature (RT; ~22 °C) Earle’s salt solution supplemented with 10 mM HEPES (pH = 7.4). The slices were then digested with trypsin (0.25%; Invitrogen, Carlsbad, CA) for 20 minutes at RT with frequent agitation of the solution. The slices were then rinsed twice with RT Leibovitz’s L-15 media (Invitrogen; Anderson et al. 2004) and triturated with a fire-polished pasteur pipette. The cell-containing solution was then transferred to 35 mm culture dishes (tissue culture treated; Corning # 430165) and supplemented with a penicillin / streptomycin solution (1%; Invitrogen) and B-27 nutritional supplement (1%; Invitrogen). The plated cells were then incubated overnight at 35 °C in a humidified air environment. These culture conditions were optimized to reduce the formation of processes and at the same time promote healthy cells suitable for assaying voltage-gated sodium channel function. All experimental protocols used in this study followed the guidelines set by the Canadian Council on Animal Care and the University of Manitoba.

Electrophysiology

After approximately 18-24 hours of incubation the culture media was replaced with the external recording solution designed to isolate sodium currents. The culture dishes were then placed directly on the stage of an inverted Nikon Diaphot 300 inverted microscope with a Nikon UV-2A filter set. To limit potential phototoxic damage to labeled cells, large, healthy looking, round cells were first identified under bright-field
illuminated, then quickly exposed to the fluorescent light to determine if labeling was present (Fig.1B). It is interesting to note that many large cells were identified in these cultures that lacked the fluorescent label and therefore recordings were not made from these cells. This observation is consistent with our previous observations (Huang et al. 2000; Carlin et al. 2006) as well as the observations of others (Thurbon et al. 1998), of large non-motoneuronal cells in the lumbar cord. Whole-cell recordings were made from Fluoro-Gold-positive cells using an Axopatch 1D amplifier, DigiData1200 A/D converter and Clampex 8 software (Axon Instruments, Union City, CA, USA). A holding potential of -70 mV was used for all experiments. This potential was used as it was well-tolerated by the cells and it likely produced a pseudo-physiological amount of steady-state sodium channel inactivation, thereby increasing the physiological relevance of these data. Data was captured at 50 kHz and lowpass filtered at 5 kHz. The borosilicate glass pipettes had resistances between 1.5 and 2.8 MΩ when filled with intracellular solution. Series resistance was compensated as required so that the calculated voltage error did not exceed 6 mV. Using a flowing KCl electrode (Neher, 1992), a liquid junction potential of -4 mV was determined, which made the true potentials more negative than those reported. All experiments were performed at room temperature (~ 22°C). For current traces in figures, depolarizing and hyperpolarizing capacitive transients have been either removed or truncated for clarity.

Solutions and chemicals

The intracellular solution contained (in mM): CsMeSO₃ (125), TEA-Cl (30), NaCl (5), MgCl₂ (1), CaCl₂ (0.5), HEPES (10), EGTA (10) Mg-ATP (3) and GTP (0.3). The solution pH was adjusted to 7.2. The extracellular solution contained (in mM): NaCl (124), TEA-Cl (30), MgCl₂ (2), CaCl₂ (2), HEPES (10), 4-AP (4), CsCl (3), glucose (10), KCl (3), CdCl₂ (0.3), NiCl₂ (0.1). The solution pH was adjusted to 7.4. Earle’s salt solution contained (in mM): NaCl (117.24), KCl (3.96), NaHCO₃ (26.19), NaH₂PO₄ (0.522), HEPES (10). The solution pH was adjusted to 7.4. All chemicals were purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, Ontario).
Data analysis

Current traces were analyzed using Clampfit v.9 software (Axon Instruments). Statistical analysis and curve fitting were performed with Microsoft Excel, Microcal Origin 6.0 and SigmaPlot 9.0 (Systat) software programs. Activation curves were constructed using the calculated sodium reversal potential of 83 mV. Both the activation and steady-state inactivation conductance data were fitted with a single Boltzmann equation of the form; $G/G_{\text{max}} = G_{\text{min}} + (G_{\text{max}} - G_{\text{min}}) / (1 + \exp((V_{1/2} - X) / k))$, where $G_{\text{max}}$ is the maximal conductance value, $G_{\text{min}}$ is the minimal conductance value, $k$ is the slope factor and $V_{1/2}$ is the voltage of the half maximal conductance value. Recovery and closed-state inactivation data were fitted with double exponential functions of the form; $y = A1*(1-\exp(-x/t1))+A2*(1-\exp(-x/t2))$; where $t1$ and $t2$ are the fast and slow time constants. These curves were compared by taking the mean of the raw normalized data with the exclusion of the first point over the time period indicated in the text.

Parametric statistics were used to assess differences between age groups if the data was normally distributed (Kolmogorov-Smirnov test for normality) and groups were of equal variance. If these criteria were not met, groups were compared using the Kruskal-Wallis One Way ANOVA on ranks. Cell capacitance was determined from the mean of 10-18 current transients integrated over 1 ms divided by the 10mV step that elicited the transients. Current density was obtained by dividing the cell capacitance by the peak current elicited by a voltage step to $-10$ mV from a 100 ms $-120$ mV hyperpolarization.
RESULTS

The data from 111 Fluoro-Gold-positive postnatal spinal motoneurons were analyzed in the present study. These cells spanned the postnatal period from day 1 to 12 and were divided into 4 age groups consisting of 3 days each (Fig. 1C). The mean cell capacitance (ANOVA on ranks; P = 0.41), and the current density of the 4 age groups were not significantly different (ANOVA; P = 0.74; Table 1). This permitted an accurate comparison of the currents across the age groups without a differential voltage error affecting the results.

Voltage-dependence of channel gating

As a first step in exploring developmental changes in sodium channel functioning we examined the voltage-dependence of activation or inactivation of channel gating as these parameters can have dramatic effects on channel availability and gating at a given membrane potential. The voltage-dependence of activation and fast inactivation of the sodium channels was measured using standard voltage protocols (Fig. 2A,B). Both the activation and inactivation conductance-voltage relationships were fitted well with a single Boltzmann function. The average V1/2 values and slope (k) values of both the activation and inactivation fits were not significantly different across the measured developmental period (Table 1). Given the remarkable consistency of these parameters across the age groups, an average of the full data set was generated and is illustrated in Fig. 2C. This analysis resulted in an average V1/2 of activation of -21.3 mV (k = 4.6) and V1/2 of inactivation of -51.5 mV (k = 7.0) that describes the voltage-dependence of sodium channel gating in spinal motoneurons during this postnatal period. The highly consistent data obtained from motoneurons over this developmental period indicates that a change in the voltage-dependence of activation or fast inactivation does not contribute to the described developmental increase in excitability of these cells.
**Table 1.** Biophysical properties of mouse spinal motoneurons and their voltage-dependent sodium current over the first 12 postnatal days. Cells were dissociated and placed in short-term (18-24 hrs) culture. No significant differences (ns) were detected in the values across age groups for any of the parameters. Values are mean +/- SD with the number of cells indicated in parentheses.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P 1-3</th>
<th>P 4-6</th>
<th>P 7-9</th>
<th>P 10-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacitance (pF)</td>
<td>9.55 +/- 3.7 (26)</td>
<td>9.97 +/- 5.5 (33)</td>
<td>9.23 +/- 5.1 (28)</td>
<td>10.39 +/- 4.0 (24)</td>
</tr>
<tr>
<td>Current density (pA/pF)</td>
<td>188 +/- 73 (17)</td>
<td>182 +/- 126 (17)</td>
<td>168 +/- 109 (15)</td>
<td>205 +/- 131 (18)</td>
</tr>
<tr>
<td>V\textsubscript{1/2} activation</td>
<td>-20.9 +/- 4.4 (23)</td>
<td>-22.6 +/- 4.7 (24)</td>
<td>-21.0 +/- 4.2 (20)</td>
<td>-19.1 +/- 4.1 (21)</td>
</tr>
<tr>
<td>Slope factor (k)</td>
<td>4.5 +/- 0.8 (23)</td>
<td>4.5 +/- 0.6 (24)</td>
<td>4.6 +/- 0.4 (20)</td>
<td>4.1 +/- 0.6 (21)</td>
</tr>
<tr>
<td>V\textsubscript{1/2} inactivation</td>
<td>-52.9 +/- 5.7 (17)</td>
<td>-52.7 +/- 6.7 (16)</td>
<td>-52.0 +/- 4.0 (14)</td>
<td>-50.4 +/- 5.4 (19)</td>
</tr>
<tr>
<td>Slope factor (k)</td>
<td>6.6 +/- 0.9 (17)</td>
<td>6.2 +/- 0.9 (16)</td>
<td>6.4 +/- 1.1 (14)</td>
<td>6.2 +/- 0.9 (19)</td>
</tr>
</tbody>
</table>

In order for a cell to fire multiple action potentials a sufficient number of sodium channels need to remain available before each spike. To assess channel availability during repetitive stimulations, such as would occur during a period of repetitive action potential firing, we assessed the sodium current amplitude during various frequencies of stimulation. To do this, cells were depolarized for 20 ms from -70 mV to -10 mV x 20 pulses at 1, 10, 20 and 30 Hz (Fig. 3A). It was found that the sodium current amplitude was reduced in a frequency-dependent manner, with the degree of inhibition by the 20\textsuperscript{th} pulse increasing as the frequency of stimulation increased. This was consistent across the age groups (Fig. 3B). Also across all age groups and at frequencies greater than 1 Hz, the current exhibited an initial rapid reduction in amplitude followed by a more gradual reduction. The rate of the initial reduction was almost identical across the age groups – consistent with the comparable fast-inactivation process seen across age groups (Table 1). The latter slower component likely represents channels being driven into a slow-inactivated state (Rush et al. 1998; Blair and Bean, 2003). As depicted in the insets in Fig. 3B the current from the P10-12 age group consistently had the slowest transitions into this slow-inactivated state across all frequencies.
When assessing the average channel availability over the 20 pulse stimulations, significant differences were noted between age groups. For cells in the first three age groups (comprising cells P1 – P9), sodium channels demonstrated an increasing tendency to accumulate in an inactivated state, leading to a progressive reduction in channel availability during all stimulation frequencies (Fig. 3C). This trend was reversed in the oldest age group (P10-12). As hypothesized, the most mature cells were on average significantly less susceptible to current loss compared to the younger age groups during the repetitive stimulation protocols. These results indicate that the developmental change in sodium channel availability is likely an important factor in the development of mature firing capabilities. Unexpectedly, however, we found that the youngest cells were also capable of maintaining a reasonable amount of available channels by the 20th pulse for all stimulation frequencies.

Recovery from inactivation

Having excluded differences in the voltage-dependence of the channels (Table 1), the ability to maintain current during repetitive stimulations is likely a consequence of either an ability of the channels to quickly recover from an inactivated state (Bean et al. 1983) or an ability to resist entering an inactivated state. These two possibilities were investigated further to understand the factors permitting the more mature cells to better follow repetitive stimulations without loosing current.

The ability to recover from the inactivated state, or “reprime” (Bean et al. 1983) was assayed using a double-pulse protocol with an increasing interval (Fig.4A). The data for the recovery process in these cells was best fitted with two exponentials (see legend of Fig. 4 for values) suggesting that even with a short 20 ms stimulation a small percentage (∼ 20%) of channels transition into a slow-inactivated state. As can be seen from the averaged data in Fig. 4B, significant differences between age groups could be seen in both the rate at which the channels recovered as well as in the completeness of recovery over the 300 ms assayed. Given longer intervals, such as the 1000 ms interval used in the 1 Hz stimulation protocol (Fig. 3B), channels in all age groups tended to become equally
available (~95%). Currents elicited from cells in both the youngest (P1-3) and oldest (P10-12) age groups tended to have a greater proportion of channels recovering in the fast phase of recovery and trended to transition into a slow-inactivated state to a lesser degree than the two middle age groups.

These trends within the data became more obvious when we assessed the change in channel availability over various time intervals of the recovery process. A change in the channel availability during the first three time intervals (50 ms, 100 ms and 150 ms) provided an indication of the rate of recovery while comparing channel availability over the last 100ms (200 – 300 ms of the protocol) permitted a comparison of the tendency to remain in a slow-inactivated state. Significant differences between groups could be seen in the first 50 ms interval data. This interval encompasses the intervals used in both the 20 Hz (50 ms) and 30 Hz (33 ms) stimulation protocols and mainly reflects the population of channels in a fast gating mode (Morgan et al. 2000) or fast phase of recovery (Bean et al. 1983). Here the P10-12 age group showed significantly greater recovery than cells of the P7-9 age group. As the interval length was increased (100 ms and 150 ms), these significant differences in channel availability extended to all the younger age groups. As seen in Fig. 4C, a “U-shaped” pattern emerged over the developmental period spanned in this study - similar to the pattern in the repetitive stimulation data.

A similar pattern was also seen in the data assessing the population of channels in the slow phase of recovery – those channels that tended to remain in a slow-inactivated state (Fig. 4D). Here again the oldest age group (P10-12) had significantly greater channel availability compared to the younger age groups (P < 0.05; one-way ANOVA on ranks). Again the oldest and youngest age groups were similar and a “U-shaped” pattern was seen. The similarity between the pattern of the age-dependent changes in frequency-dependent inhibition (Fig. 2C) and both the fast and slow components of the recovery from inactivation (Fig. 4C,D) suggested that these two parameters were related. This relationship was explored further below.
Correlation analysis

Thus far the data has been arranged into age groups in order to examine the changes that occur during postnatal development. At this point we wished to examine the underlying factors that allowed cells to maintain available channels during repetitive stimulations. For this we grouped cells from all age groups together, as some cells in all the age groups showed a remarkable ability to follow high frequencies even though on average the cells in the oldest age group were the most proficient at this task. At all frequencies studied, a strong and highly significant correlation existed between the degree of frequency-dependent inhibition (taken to be the amplitude of the 20\textsuperscript{th} pulse divided by the amplitude of the first pulse) and channel availability over the initial 50 ms interval of recovery (Fig. 5A). The data for the 100 ms (0.62 < R< 0.93; P< 0.0001; n = 58 cells) and 150 ms (0.86< R< 0.91; P < 0.0001; 58 cells) intervals showed similarly strong and highly significant correlations at all frequencies (Pearson Product Moment Correlation; data not shown).

On a cell-by-cell basis there were also highly significant (P < 0.0001) correlations between the degree of frequency-dependent inhibition and the current recovery over the 200 – 300 ms interval (Fig. 5B). As this interval is entirely within the slow phase of recovery it represents the ability of channels to either transition from or resist entering a slow-inactivated state. These strong correlations between the ability of a cell to maintain channel availability during repetitive stimulations and the recovery of channels from inactivation provide evidence that the process of channel “repriming” is an important factor in the ability of the spinal motoneuron to fire multiple action potentials. This factor has been suggested to be important for permitting high firing rates in other cell types (Cummins et al. 1998, 2001; Spampanato et al. 2001; Herzog et al. 2003).

Closed state inactivation

Because sodium channels do not need to actually open in order to transition into an inactivated state (Taddese and Bean, 2002), we assessed the potential contribution of closed-state inactivation to the observed changes during repetitive stimulations. For this assessment the membrane potential was depolarized 20 mV more positive to the holding potential for varying periods of time before a test pulse was used to assess channel
availability. The initial depolarization to -50 mV did not cause channel activation (Fig. 6A). Current amplitudes were normalized to the amplitude of the first evoked current.

As shown in the graph in Fig. 6B, the current in all of the age groups quickly (within 200 – 400 ms) decreased (fast component) and thereafter reached a similar rate of decay (see slope values in legend), likely that of a second slower inactivation process (slow component). Here again obvious age-related differences in channel availability could be seen at any given time point. Interestingly, with respect to closed-state inactivation it was the P4-6 age group that demonstrated the greatest ability to maintain current under these test conditions. At present it is unclear why the current in this age group was so resistant to this particular transition. Significant differences between the age groups were seen when the average channel availability during the protocol is compared (Fig. 6C). Here the average current resistant to closed-state inactivation in the P4-6 age group was significantly greater than the other three groups (P < 0.05). As well, the current in the P10-12 group was significantly more resistant to closed-state inactivation than the P7-9 group (P < 0.05). With the exception of the P4-6 data, these data demonstrated a similar trend as the repetitive stimulation data. With respect to this biophysical parameter, cells of the P10-12 age group were very similar to the P1-3 age group and more resistant to inactivation than the P7-9 age group.

**Correlation analysis**

Similar to the variability in the repriming data, the ability to resist closed-state inactivation in some cells was not representative of the average of their age group. Therefore we looked at individual cells to determine if there was a correlation between the ability to retain current during repetitive stimulations and the closed-state inactivation process (Fig. 7). At 10 Hz the correlation just reached significance (P = 0.049) while at 20 Hz and 30 Hz these two parameters showed more significant relationships (P = 0.001 and 0.002 respectively). This strong correlation suggests that a component of the cell’s ability to maintain available channels is also related to the fast component of closed-state inactivation, as this fast process determined the relative channel availability for depolarizations up to 2 seconds. That is, the slow inactivation process was essentially equal across the groups (ie. similar slope values). The age-dependent pattern seen in Fig.
and the significant correlation between closed-state inactivation and the ability of channels to resist entering an inactivated state during repetitive stimulations suggests that inactivation from the closed state also plays an important role in the developmental change of motoneuronal firing patterns.

Open-state inactivation

With clear age-related changes in both the closed-to-inactivated and the inactivated-to-closed-state transitions we assessed whether there were also age-related differences in the open-to-closed state transitions. The current decay during a voltage step could be fitted with a single exponential function and was plotted relative to membrane potential (Fig. 8). When comparing the rate of inactivation from the open-state of the channel a significant difference between the age groups could not be detected at any voltage examined. There was greater scatter between the means at the more hyperpolarized voltages but even at a membrane potential of –30 mV this was not significant (ANOVA on ranks; P = 0.37). Furthermore, there was not a significant correlation between the rate of open-state inactivation and the ability to follow repetitive stimulations (10 - 30 Hz) when assessed on a cell-by-cell basis (R < 0.11 and P > 0.46; data not shown).
DISCUSSION

In the present study we assessed the changes in the biophysical properties of the isolated voltage-gated sodium current in spinal motoneurons over the first 12 postnatal days. We demonstrated that the voltage-gated channels mediating this current undergo a pattern of changes such that the channels expressed in the most mature cells are better able to maintain channels in an available state during repetitive stimulations. This finding is consistent with the hypothesis that a developmental change in the biophysical properties of sodium channels contributes to the well described developmental increase in firing rate of these cells. A developmental increase in the ability to maintain channels in an available state would work synergistically with the other known developmental changes in these cells, such as the increase in sodium current density (McCobb et al. 1990; Garcia et al. 1998; Gao and Ziskind-Conhaim, 1998) and hyperpolarizing conductances (McCobb et al. 1990; Vinay et al. 2000), to enable the mature motoneurons to discharge at high frequencies for extended durations.

The increased ability of the most mature cells to maintain channels in an available state appears to be derived mainly from the ability of the channels to recover quickly from an inactivated state – firstly because of the highly significant correlation that was seen between these two parameters and secondly because the developmental pattern of changes in this biophysical parameter paralleled those seen with frequency-dependent inhibition. It is important to note that the developmental change in the ability to follow repetitive stimulations was seen in the absence of an age-related change in the voltage-dependence of channel gating as a hyperpolarizing shift in this parameter relative to the holding voltage could also result in a greater availability of channels.

The tight association between the ability to maintain available channels during repetitive stimulations and the ability to quickly recover from inactivation is not unexpected. This relationship has been demonstrated in previous studies (Bean et al. 1983; Ilyin et al. 2005) and in fact is thought to be an important component of the mechanism of action of many sodium channel inhibiting drugs (ex. lidocaine, phenytoin, carbamazepine). Although the ability to reprime quickly will enable the current to more faithfully follow repetitive stimulations this ability does not necessarily endow channels
with the ability to maintain current during repetitive stimulations. For instance, when Na$_{\text{v}1.2}$ and Na$_{\text{v}1.6}$ channels were compared after expression in sensory neurons, the Na$_{\text{v}1.2}$ isoform was found to display faster repriming kinetics than did the Na$_{\text{v}1.6}$ isoform, even though it was the Na$_{\text{v}1.6}$ expressing cells that were better able to maintain current during repetitive stimulations (Rush et al. 2005). Additionally, when Zhou and Goldin (2004) co-expressed these same two isoforms with the β1 subunit in *Xenopus* oocytes they also observed that the Nav1.6 isoform maintained significantly more current during repetitive stimulations than did the Nav1.2 isoform. However, under these experimental conditions the Nav1.6-mediated current was actually *potentiated* during repetitive stimulations while the Nav1.2-mediated current showed the typical decline. Clearly this difference could not be due to isoform differences in repriming kinetics, and in fact was attributed to differences in the activation process that occurred during the repetitive stimulations (Zhou and Goldin, 2004).

In the present study the ability of spinal motoneurons to maintain channels in an available state seems to receive contribution from differences in other state transitions in addition to the transition from the inactivated-to-closed state (ie. repriming). As demonstrated in Fig.3B, the channels in the most mature age group consistently had a slower transition rate into a slow-inactivated state compared to the other age groups (see Fig. 3B insets). This tendency to either resist transitioning into a slow-inactivated state or recover from this state quickly was also evident during the repriming experiments (Fig.4B). In these latter experiments the current in the mature cells showed a greater degree of recovery over the last 100 ms of the protocol. Interestingly, the transitions into a slow-inactivated state from the closed state were very similar across the age groups (similar slope values; Fig. 5B) and therefore may represent transitions into a different slow-inactivated state. Finally, a decreased tendency to undergo the closed-to-inactivated state transition correlated well with the ability to maintain available channels during repetitive stimulations. That is, with fewer channels participating in the fast phase of closed-state inactivation, the more channels were available for a subsequent opening. These finding suggest that differences in these inactivation transitions also contribute to the ability to follow repetitive stimulations without loosing appreciable current.
The occurrence of closed-state inactivation during the repetitive stimulation protocols at first may seem unusual but these cells were assessed from a holding potential of $-70\text{mV}$. From the inactivation conductance-voltage graph (Fig. 2) this voltage corresponds to roughly 10% of channels being inactivated. Therefore, holding at this potential one would expect to see transitions to an inactivated state and an increase in the rate of these same transitions as the membrane potential was pulsed to more depolarized potentials where channel openings occurred (Nuss et al. 2000).

A surprising finding in this study was that the current in the youngest cells were also very proficient at following repetitive stimulations and with respect to other parameters, behaved in a similar manner to the most mature cells. A possible explanation for this will be discussed in the following section but this finding suggests that, at least for the very early postnatal cells, the biophysical properties of the voltage-gated sodium current do not limit the maximal firing rates. Other mechanisms such as the low sodium current density (McCobb et al. 1990; Garcia et al. 1998; Gao and Ziskind-Conhaim, 1998), or the under-developed potassium (McCobb et al. 1990; Vinay et al. 2000) or other conductances (McCobb et al. 1989; Mynlieff and Beam, 1992; Miles et al. 2004) likely play a larger role in limiting the maximal firing rate at this stage of development.

Plateau potentials

Mature spinal motoneurons generate plateau potentials (Perrier and Hounsgaard, 2000), a quasi-stable potential approximately 10 - 20 mV more positive to the resting potential (Hounsgaard et al. 1986; Hounsgaard and Kiehn, 1989), which is capable of eliciting repetitive action potential discharges. In the present study we sought to identify a possible means whereby sodium channels could remain available to discharge multiple action potential under depolarized conditions which normally promote transitions into an inactivated and therefore unavailable state. To accomplish this we tested the ability of motoneuronal sodium channels to resist transitioning from the closed-state to the inactivated-state using a voltage protocol that mimicked a plateau potential (Fig. 6A). In these experiments the most mature cells were better able to resist transitioning into the inactivated state than the P7-9 age group (but it is unclear as to why the P4-6 age group was so proficient at this particular task). Regardless, should this trend in closed-state
inactivation continue, this biophysical parameter would likely become important for the expression of the mature firing properties of spinal motoneurons. This late-developing resistance to inactivation may contribute to the difficulty of demonstrating plateau potentials in spinal motoneurons of rodents until well into the second postnatal week (Jiang et al. 1999c). Furthermore, postnatal development of the resistance to closed-state inactivation would likely contribute to previous results suggesting that nifedipine-sensitive plateau potentials play in increasing role in lumbar ventral root output during postnatal development (Jiang et al. 1999b).

**Developmental changes in channel subtypes**

Previous studies have demonstrated that the complement of voltage-gated sodium channels expressed in the spinal cord in general, and specifically in motoneurons, changes during postnatal development. Channel expression assessed at both the protein (Gordon et al. 1987; Schaller and Caldwell, 2000) and mRNA levels (Beckh et al. 1989; Black et al. 1994; Felts et al. 1997; Garcia et al. 1998; Schaller and Caldwell, 2000) in the rodent spinal cord have demonstrated that around birth the sodium channel subtypes Na\(_{\text{v}}\)1.1, 1.2, 1.3 and 1.6 are all expressed to varying degrees. There is some discrepancy as to the prominent channel subtype between studies but there is consensus that over the first two postnatal weeks there is a relative change in the expression of these channels. With respect to the ventral horn cells in particular, at birth these cells express detectable levels of Na\(_{\text{v}}\)1.1, Na\(_{\text{v}}\)1.2 and Na\(_{\text{v}}\)1.3 mRNA transcripts (Felts et al. 1997). During the following two weeks of development the levels of Na\(_{\text{v}}\)1.1 mRNA are seen to increase, the levels of Na\(_{\text{v}}\)1.3 decrease while the levels of Na\(_{\text{v}}\)1.2 are seen to remain somewhat stable (Felts et al. 1997). The expression of Na\(_{\text{v}}\)1.6 mRNA (Felts et al. 1997; Schaller and Caldwell, 2000) and protein (Schaller and Caldwell, 2000) in motoneurons becomes detectable between P2 and P7 and increases until approximately the third postnatal week. These labeling studies are in good agreement with electrophysiological data obtained from the Na\(_{\text{v}}\)1.6 mutant mouse (*med*; Garcia et al. 1998). In this study the authors demonstrated that in the absence of the Scn8a gene encoding the Na\(_{\text{v}}\)1.6 channel, the normal postnatal increase in current density of spinal motoneurons failed to occur despite normal current density at P0. These data support the suggestion that the Na\(_{\text{v}}\)1.6 channel
isoform becomes the major current contributor in these cells by the second postnatal week.

**Heterolgous expression and function of channel subtypes**

The biophysical properties of most of the mammalian sodium channel α subunits (with or without accompanying β subunits) have been studied in relative isolation after their expression in various heterologous systems. These studies have demonstrated the fundamental properties of the four main sodium channels expressed in spinal motoneurons (Smith et al. 1998; O'Leary, 1998; Cummins et al. 2001; Zhou and Goldin, 2004). Of those channels that would be expressed during the first days after birth, Na\(_{v}\)1.1 and Na\(_{v}\)1.2 channels appear to have very similar properties. When expressed under similar conditions (*Xenopus* oocytes with the same β subunits), the voltage-dependence of activation and inactivation were similar with Na\(_{v}\)1.2 having slightly more hyperpolarized values. Both of these channels are capable of recovering from inactivation quickly (fast tau < 2.2 ms) and with greater than 80% recovering in during the fast time constant (Smith *et al.* 1998). Again, when compared under similar expression conditions (HEK-293 cells), the voltage dependence of activation and inactivation of Na\(_{v}\)1.3 channels were found to be within 4 mV of the Na\(_{v}\)1.2 channels (O'Leary, 1998; Cummins *et al.* 2001). The Na\(_{v}\)1.3 isoform was found to recover from inactivation three times slower than the Na\(_{v}\)1.2 isoform at -80 mV. On the other hand, at -60 mV the Na\(_{v}\)1.3 channels were found to undergo closed-state inactivation more than two fold slower.

The fourth channel isoform expressed in these cells during this developmental period is Na\(_{v}\)1.6. When Na\(_{v}\)1.6 channels were co-expressed with the β1 subunit in *Xenopus* oocytes, these channels were so proficient at remaining in an available state that repetitive stimulations elicited a potentiation of this current (Zhou and Goldin, 2004). In the same study the Na\(_{v}\)1.2 isoform showed a modest decrease in the current. Moreover, when Na\(_{v}\)1.6 was compared to Na\(_{v}\)1.2 after expression in dorsal root ganglion cells, the Na\(_{v}\)1.6 isoform was significantly better at resisting inactivation during repetitive stimulation protocols (Rush *et al.* 2005).

The most parsimonious explanation for the main finding of this paper, the fact that the older cells can retain more current during repetitive stimulations, is a postnatal
increase in the expression of Na\textsubscript{v}1.6 – a channel subtype that has been demonstrated to be able to retain available channels during this type of stimulation. This finding is supported by studies demonstrating that deletion of the Scn8 gene encoding the Na\textsubscript{v}1.6 channel results in a decreased maximal firing rate of cells that normally express this isoform (Van Wart and Matthews, 2006; Levin et al. 2006; Enomoto et al. 2007). Moreover, absence of this channel isoform in trigeminal neurons of the Na\textsubscript{v}1.6 null mice resulted in a reduced ability to maintain repetitive discharges compared to wild-type littermates (Enomoto et al. 2007). These authors recognized that this was consistent with the complement of Na\textsubscript{v}1.6-containing wild-type channels being less susceptible to, and faster at recovering from inactivation.

Changes in β-subunit expression

The expression of the various accessory β subunit isoforms also changes during early development. Co-expression of these β subunits has been shown to have a strong influence on various biophysical parameters of the expressed α subunit mediated currents (Isom et al. 1992). During the embryonic period high levels of the β3 mRNA transcript are detected in the rat CNS. After P1 these levels continually decline (Shah et al. 2001). On the other hand levels of β1 transcripts are first detected at approximately P3 and increase until approximately P14 while β2 mRNA appears at approximately the same time and the levels increase until reaching adulthood (Shah et al. 2001). All three α subunit isoforms that predominate during the first few days of postnatal development, Na\textsubscript{v}1.1, Na\textsubscript{v}1.2 and Na\textsubscript{v}1.3, can all form complexes with β3. With respect to Na\textsubscript{v}1.2 (Morgan et al. 2000) and Na\textsubscript{v}1.3 (Shah et al. 2001) the addition of this subunit increases the degree of channel availability during recovery from inactivation. Under these conditions young cells may be expected to retain a large fraction of available channels during repetitive stimulations as seen in the present study.

The above discussion on the postnatal changes in both the sodium channel α and β isoforms, and their expected functional effects on the sodium current in spinal motoneurons, provides a possible explanation for the “U-shaped” developmental pattern observed in this study. During the earliest postnatal period the cells would express a
combination of Na\textsubscript{v}1.1, Na\textsubscript{v}1.2 and Na\textsubscript{v}1.3 channels in the presence of \(\beta3\) subunits. This would permit all of these channels to recover from inactivation relatively fast and likely contribute to the ability of the current to resist inactivation during the repetitive stimulations protocol. With further development, while the level of Na\textsubscript{v}1.1 increases, that of the relatively fast repriming Na\textsubscript{v}1.3 and \(\beta3\) combination decreases, as there is a change from \(\beta3\) to \(\beta1\) and \(\beta2\). Moreover, during this period the fast repriming Na\textsubscript{v}1.2 channels contribute a smaller fraction to the total current as the overall current density in these cells increases. These changes likely result in an overall decrease in the ability of the cell to retain current during repetitive stimulations. With the increase of the Na\textsubscript{v}1.6 isoform during the second postnatal week, the ensemble current would again acquire the ability to retain available channels during repetitive stimulations, as this \(\alpha\) subunit isoform appears to be the most proficient at this task.

**Limitations**

An important factor in this present work was the ability to maintain voltage control over the sodium current. This is a difficult task in spinal motoneurons due to the large current density in these cells and the fact that these channels are clustered at the initial segment and in the axon – structures spatially remote from the somatic pipette during whole-cell recording. In the present work we sought to circumvent these voltage- and space-clamp problems by removing the motoneuronal processes through mechanical isolation of the soma and then culturing these cells in conditions that did not promote process growth (absence of growth factors, uncoated plastic substrate). The cells were exposed to these culture conditions for a relatively short period of time with the goal of replicating the in-vivo channel expression as previously described (Garcia et al. 1998). As discussed below, these experimental procedures are both a strength and a possible limitation of the present work.

With the use of these experimental conditions the cells had their initial segment removed and subsequently re-expressed their sodium channels. We believe that the recorded currents are from newly expressed channels for two main reasons: 1) cells patch-clamped immediately after trituration did not demonstrate a sodium current (data not shown) and 2) given the same incubation time, cells of the various age groups
demonstrated similar current densities (Table 1). It is not surprising that the cells did not demonstrate a sodium current after mechanically removing the processes as a similar result was seen when the same process-removal effect was accomplished by pulling the intact soma away from the rest of the cell in a transverse slice of spinal cord tissue (Safronov et al. 2000). In this case, the sodium current density in the soma-only situation was reduced dramatically compared to the current recorded from the intact cell. The ability to re-express the sodium channels in isolated somata has also been demonstrated. The cell bodies of the squid giant axon are normally unexcitable, but if dissociated from the axon and cultured these cell bodies will begin to develop an inward sodium current that begins on the day of isolation and increases in density during the culture period (Brismar and Gilly, 1987). Moreover, the vast majority of these newly synthesized channels are biophysically indistinguishable from the “normal” axonal channels (Gilly and Brismar, 1989). To definitively determine whether or not the complement of newly-expressed sodium channels in the present study are the same as would be expressed in the in age-appropriate in-vivo cells will require either evaluation of the protein or mRNA. But the fact that the sodium current in these cells demonstrated age-related changes in inactivation properties and that these inactivation changes are consistent with the known maturation of firing properties in these cells strongly suggests that the expression of the age-appropriate channels occurred in these cells. Nonetheless, we cannot discount the possibility that an age-related change in some intracellular modulatory factor also plays a role in the observed changes.

In the present study we isolated and examined one small component of the much more complex intact spinal motoneuron. We view this culture preparation as a model of an intact in-vivo motoneuron and as with any reduced preparation there is a trade-off between gaining control in one aspect (in this case voltage control) and the loss of other aspects of the original condition. Our goal was to create an experimental condition that allowed a very accurate study of the sodium current in motoneurons and in doing so we may have given up the ability to assess other aspects of motoneuron functioning. Although we did not attempt to assess action potential firing, in all likelihood the somata examined in the present study would not discharge as motoneurons would in a more intact preparation (ex. slice, whole cord or in-vivo animal). The motoneurons examined
in the present experiments lacked some of the fundamental components of spinal motoneurons such as the dense sodium channel clustering on the initial segment (Alessandri-Haber et al. 1999) and not only the dendritic membrane itself (which can have an effect on firing patterns; Mainen and Sejnowski, 1996) but also the dendritic conductances important for plateau potential generation (Lee and Heckman, 1998; Carlin et al. 2000b). Furthermore, the overall sodium current was many fold lower under the present experimental conditions compared to that recorded from more intact spinal motoneurons (ex. Miles et al. 2005). As such, the degree to which the presently-described changes in sodium channel functioning manifest themselves in the behavior of intact motoneurons during development has yet to be fully defined.

Functional consequences

In the present study we have demonstrated that the most mature spinal motoneurons possessed the greatest ability to follow repetitive stimulations due to their ability to maintain channels in a state capable of opening and passing current. This ability stems from age-related changes in various channel transitions but results mainly from an increased ability of the channels to recover from inactivation quickly. The age-related decrease in the tendency of these channels to transition into a slow-inactivated state or undergo closed-state inactivation also contributes to maintaining available channels during repetitive stimulations. The functional consequence of these kinetic changes in the native cells would be amplified by the well-described developmental increase in the sodium current density (McCobb et al. 1990; Garcia et al. 1998; Gao and Ziskind-Conhaim, 1998). Therefore, these cells would not only have the benefit of a larger pool from which to draw available channels, but these channels would also tend to remain in an available state. These two factors likely greatly impact the ability of these cells to fire repetitively for extended periods of time.
REFERENCES


Felts PA, Yokoyama S, Dib-Hajj S, Black JA and Waxman SG. Sodium channel alpha-subunit mRNAs I, II, III, NaG, Na6 and hNE (PN1): different expression


**Garcia KD, Sprunger LK, Meisler MH and Beam KG.** The sodium channel Scn8a is the major contributor to the postnatal developmental increase of sodium current density in spinal motoneurons. *J Neurosci* 18: 5234-9, 1998.


Mainen ZF and Sejnowski TJ. Influence of dendritic structure on firing pattern in


**Nuss HB, Kambouris NG, Marban E, Tomaselli GF and Balser JR.** Isoform-specific lidocaine block of sodium channels explained by differences in gating. *Biophys J*


Shah BS, Stevens EB, Pinnock RD, Dixon AK and Lee K. Developmental expression


ACKNOWLEDGEMENTS

The authors wish to thank Drs. G. Wu, V. Ilyin and M. Fry for their helpful comments on the manuscript, Carolyn Gibbs for her technical assistance and Maria Setterbom for assistance with figure preparation. This research was supported by grants to LMJ from the Canadian Institutes for Health Research (#37755) and the National Institutes for Health (#1R01NS4090301). J. Liu was supported by a scholarship from the National Science and Engineering Research Council of Canada (NSERC).
FIGURE LEGENDS

Figure 1. Identification of spinal motoneurons in culture. A. A low-power image of an acute transverse slice of the lumbar spinal cord harvested from a P6 mouse under epifluorescent illumination demonstrating Fluoro-Gold positive cells restricted to the ventral horns of the slice (D = dorsal, V = ventral). B. A higher power bright field image of spinal cord cells after 24 hours in culture (Ba). Spinal cord slices were enzymatically digested prior to dissociation of the cells. (Bb) Same field of view as in (Ba) under epifluorescent illumination demonstrating that motoneurons can be identified in these mixed cultures. Note the absence of process formation on these cells. C. Histogram demonstrating the number of cells from each postnatal day and the age groups used in the present study.

Figure 2. Voltage-dependence of the sodium current in spinal motoneurons. A. Representative sodium currents elicited from a P12 motoneuron by step depolarizations. These currents activated in a graded and voltage-dependent manner. Voltage protocol illustrated below currents. B. Currents from the same cell as in (A) demonstrating voltage-dependent inactivation. Membrane potential was stepped from -70 mV to various potentials for 100 ms before a 20 ms test pulse to -10 mV was delivered. C. Voltage-conductance relationships describing the current during the full postnatal period studied (P1-12). Voltage of half maximal (in)activation and slope values describing these relationships were generated by fitting the averaged data with a Boltzmann function (solid lines). Activation values were: V_{1/2} = -21.3 mV; k = 4.6; n = 88 cells. Inactivation values were: V_{1/2} = -52.1 mV; k = 5.7; n = 65 cells. Mean +/- SE.

Figure 3. The isolated sodium current in spinal motoneurons shows developmental changes in the degree of frequency-dependent inhibition. A. Representative current traces recorded from a P12 motoneuron. Twenty current traces are overlaid at each stimulation frequency. The stimulation consisted of 10 ms depolarizations from -70 mV to -10 mV. B. Averaged data demonstrating the patterned change of the frequency-dependent inhibition over the first 12 postnatal days. Fractional current was defined as the fraction
of current elicited by a test pulse in the stimulus train relative to the initial pulse. Data were fitted with a double exponential function. Each point represents data from: 1 Hz; 15-16 cells, 10 Hz; 21-25 cells, 20 Hz; 15-16 cells, 30 Hz; 20 cells. Mean +/- SE. Slow inactivation time constants for the various age groups are shown in the insets. C. Summary chart of the mean frequency-dependent inhibition data as a function of age. The fraction of available channels was defined as the fractional current averaged over 20 pulses at the various frequencies indicated. Error bars are the 95% confidence intervals. Statistically significant (P < 0.05) differences between mean values are indicated as: * - 10 Hz, # - 20 Hz, ψ – 30 Hz (One Way ANOVA on ranks with Dunn’s multiple pairwise comparison). Note that the P10-12 group was significantly different than all other groups at 10, 20 and 30 Hz.

Figure 4. The ability of sodium channels to recover from inactivation or “reprime” changes with postnatal age. Aa. Overlaid current traces demonstrating the response of the sodium current in a P12 cell to the double-pulse voltage protocol illustrated. In this protocol, the inter-pulse duration is increased by 10 ms during successive sweeps. Ab. The first and second pulses from the current response illustrated in (Aa) overlayed on an expanded time scale. B. Averaged re-priming data from the four age groups (P1-3 n = 15; P4-6 n = 11; P7-9 n = 13, P10-12, n = 18). Data are expressed as the current amplitude of pulse 2 (P2) relative the current pulse 1 (P1). Lines are double exponential functions describing the data. Time constant values are: P1-3; τ1 = 14.4 ms, τ2 = 88.5 ms, P4-6; τ1 = 13.4 ms, τ2 = 70.4 ms, P7-9; τ1 = 17 ms, τ2 = 96.2 ms, P10-12; τ1 = 12.9 ms, τ2 = 56.8 ms. In the main graph the mean data points and error bars removed for clarity. These are shown in the two inset graphs illustrating the changes in the rate of repriming and the completeness of recovery, respectively. C. Summary graph illustrating the fraction of channels recovered over three increasing intervals from the initial stimulus (50, 100 and 150 ms). Note that the mean values reflecting channel availability during the three intervals of recovery for the P10-12 age group are significantly different from all other groups. Other significant differences between groups are illustrated in the graph (P < 0.05; ANOVA with Student-Newman-Keuls multiple pairwise comparisons). D. Summary graph illustrating the age-related change in channel availability over the
recovery interval of 200 – 300 ms. The P10-12 age group was again significantly different than all others. Other significant differences are noted with an asterisk (*; P < 0.05; One Way ANOVA on ranks with Dunn’s multiple pairwise comparisons).

**Figure 5.** The ability of the sodium current to follow various stimulation frequencies (resist frequency-dependent inhibition) is correlated with the ability of channels to recovery from inactivation. **A.** Scatter plot demonstrating the relationship between recovery from inactivation over the initial 50 ms interval (fast gating period) and the degree of frequency-dependent inactivation (20th pulse / 1st pulse) for 10, 20 and 30 Hz. Solid lines are linear regression fits. Correlations were highly significant at all three frequencies (P < E-7; 10 Hz – R = 0.65, 20 Hz – R = 0.84, 30 Hz – R = 0.9; n = 58 cells). **B.** Scatter plot illustrating a positive correlation between channel repriming over the final 200 – 300 ms interval (slow gating period) of the data in Fig. 4B and the degree of frequency-dependent inactivation (20th pulse / 1st pulse) for the three frequencies tested. Correlations were highly significant at all frequencies (P < E-17; 10 Hz – R = 0.87, 20 Hz – R = 0.91, 30 Hz – R = 0.86; n = 58 cells).

**Figure 6.** The isolated motoneuronal sodium current displays developmental changes in closed-state inactivation. **Aa.** Raw traces from a P11 cell illustrating the current response to the protocol used to gauge the susceptibility of sodium channels to transition from the closed to inactivated state. Cells were depolarized from a holding potential of -70 mV to -50 mV for varying durations before a test pulse to -10 mV was applied to assess channel availability. Twenty overlaid sweeps are illustrated. **Ab.** Current responses from the first and 20th sweep on an expanded time scale. **B.** Averaged data for the four age groups as a function of time. Currents were normalized to the initial current amplitude. P1-3; n = 11 cells, P4-6; n = 9 cells, P7-9; n = 7 cells, P10-12; n = 12 cells. Mean +/- SE. Lines are exponential-linear combination functions describing the data with time constants (τ) and slopes (m) of: P1-3 τ = 109 ms, m = -0.13/s; P4-6 τ = 43 ms, m = -0.13/s; P7-9 τ = 96 ms, m = -0.15/s; P10-12 τ = 93 ms, m = -0.14/s. **C.** Summary graph comparing the average
channel availability over 2 seconds (average of data used to construct curves in (B)) relative to postnatal age. Statistically significant (P < 0.05) differences between mean values are indicated (*; ANOVA on ranks with Dunn’s pairwise comparison). Note that the P4-6 age group was significantly different from all other groups other significant differences are denoted with an asterisk (*). Error bars are 95% confidence intervals.

**Figure 7.** The ability of channels to remain available during repetitive stimulations is correlated with the ability to resist closed-state inactivation. Scatter plot of the depolarization resistant current and the degree of frequency-dependent inactivation (20th pulse / 1st pulse). Solid lines are linear regression fits of the data. All correlations were reached statistically significant at the $\alpha = 0.05$ level. At 10 Hz the correlation just reached significance ($R = 0.34; P = 0.049$) but was stronger at 20 Hz ($R = 0.50; P = 0.002$) and 30 Hz ($R = 0.53; P = 0.001$) (n = 35 cells).

**Figure 8.** Inactivation from the open-state is not altered during postnatal development. The current decay rate was fitted with a single exponential function (inset) and plotted against test potentials from -30 mV to +30 mV. Significant differences were not detected at any potential. P1-3; n = 26 cells, P4-6; n = 24 cells, P7-9; n = 20 cells, P10-12; n = 21 cells. Mean +/- SE.
Figure 1
Figure 2
Figure 3

A.  

B.  

C.  

Fractional current

Fraction of available channels

Mean +/- SE

1 Hz 10 Hz

20 Hz 30 Hz

500 pA

6 ms

P1-3 P4-6 P7-9 P10-12

10 Hz 20 Hz 30 Hz

0.4 0.6 0.8 1.0

0.2 0.4 0.6 0.8 1.0

0 200 400 800

0 200 400 600

0 5 10 15 20

0 500 1000 1500 2000

0 250 500 750 1000

0 200 400 600 800

0 200 400 600

0 200 400 600
Figure 4

**Aa**

Baseline firing pattern with interpulse interval and pulse duration indicated.

**Ab**

A single action potential with P1 and P2 labeled.

**B**

Graph showing recovery over last 100 ms with interpulse intervals of 50, 100, 150, 200, 250, and 300 ms.

**C**

Bar graph comparing fractional recovery for different interpulse intervals.

**D**

Bar graph showing recovery over the last 100 ms for different groups.
Figure 5
Figure 6

A

Depolarization interval (ms)

0.2
0.4
0.6
0.8
1.0

0 500 1000 1500 2000

P1-3
P4-6
P7-9
P10-12

Ab

Inactivation resistant current

P1-3 P4-6 P10-12 P7-9

*  **

-70 mV

-50 mV

-10 mV

Δ t (ms)

× 20

400 pA

300 ms

B

P1

P20

Depolarization interval (ms)

0 500 1000 1500 2000

0 0.2 0.4 0.6 0.8 1.0

C

Inactivation resistant current

P1-3 P4-6 P7-9 P10-12

* "
Figure 7

Frequency-dependent inhibition (I/I)

Inactivation resistant current

0.2 0.4 0.6 0.8 1.0

0.0 0.2 0.4 0.6 0.8 1.0

10 Hz
20 Hz
30 Hz

Figure 7
Figure 8