Diversification of intrinsic motoneuron electrical properties during normal development and botulinum toxin-induced muscle paralysis in early postnatal mice

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Running title: Diversification of motoneuron properties during development

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Abstract

During early postnatal development, between birth and postnatal days 8-11, mice start to achieve weight-bearing locomotion. In association with the progression of weight-bearing locomotion there are presumed developmental changes in the intrinsic electrical properties of spinal α-motoneurons. However these developmental changes in the properties of α-motoneuron properties have not been systematically explored in mice. Here, data are presented documenting the developmental changes of selected intrinsic motoneuron electrical properties including statistically significant changes in action potential half-width, intrinsic excitability and diversity (quantified as coefficient of variation, CV) of rheobase current, after-hyperpolarization half-decay time, and input resistance. In various adult mammalian preparations, the maintenance of intrinsic motoneuron electrical properties is dependent upon activity and/or transmission-sensitive motoneuron-muscle interactions. In this study, we show that botulinum toxin-induced muscle paralysis led to statistically significant changes in the normal development of intrinsic motoneuron electrical properties in the postnatal mouse. This suggests that muscle activity during early neonatal life contributes to the development of normal motoneuron electrical properties.

Keywords: spinal cord, development, plasticity, electrophysiology
Introduction

During early postnatal development of the central nervous system, α-motoneurons in the spinal cord go through significant morphological and physiological changes (Fulton and Walton 1986; Li et al. 2005; Perrier and Hounsgaard 2000; Vinay et al. 2000b). Among the previously reported early postnatal changes in the intrinsic electrical properties of motoneurons are changes in input resistance and after-hyperpolarization (AHP) measures from rat pups (Clarac et al. 1998; Fulton and Walton 1986; Gao and Ziskind-Conhaim 1998; Seebach and Mendell 1996; Walton and Fulton 1986), and changes in conduction velocity, input resistance, AHP and voltage threshold in kittens (Cameron et al. 1991). These observations from rat and kitten preparations may not necessarily translate to the developing mouse, and with the advantage of potentially using transgenic mouse models to further explore these developmental processes, an examination of the development of mouse motoneuron electrophysiological properties is important to document.

Studies in rats (Fulton and Walton 1986; Gao and Ziskind-Conhaim 1998; Seebach and Mendell 1996) and cats (Bambrick and Gordon 1992) suggest that the full range of motoneuron diversity is not established at birth; rather, motoneuron diversification is a process that proceeds during early postnatal life. Previous work supports the hypothesis that normal movements, including weight-bearing locomotion, are contingent on the orderly recruitment of motor units, which are largely dependent on the intrinsic electrical properties of motoneurons (Burke 1981; Cope and Pinter 1995; Rossignol 1996). Therefore, we were interested in examining the intrinsic motoneuron properties and the diversification process of these properties during early postnatal
development in mice during the time period that these animals start to execute weight-bearing locomotion. We hypothesized that the behavioral change, from non-weight-bearing to weight-bearing locomotor activity would correlate with an increase in the diversity of intrinsic motoneuron electrical properties. To test this hypothesis, we measured a selected subset of intrinsic motoneuron electrical properties using whole-cell patch-clamp recording of motoneurons of lumbar spinal cord slices from two age groups, one before the onset of weight bearing locomotion, postnatal days 0-3 (P0-3), and a second set after the onset of weight-bearing locomotion, postnatal days 8-11 (P8-11). The results presented in this study show that there are significant changes in both the mean values and distribution of the intrinsic motoneuron properties selected for this study including action potential half-width, rheobase current (measure of motoneuron excitability), AHP half-decay time (contributes to firing rate regulation) and input resistance.

Previous studies using adult rat and cat preparations (Foehring and Munson 1990; Nakanishi et al. 2005; Pinter et al. 1991), have shown that intrinsic motoneuron electrical properties respond to changes in signaling interactions between motoneurons and muscle fibers. These studies suggest that interrupting a posited retrograde activity-sensitive signal from muscle fibers back to motoneurons decreases the mean values of rheobase current in the innervating motoneurons. However, it is not clear whether this system contributes to the early sculpting of motoneuron properties during the onset of weight-bearing locomotion. Therefore, we hypothesized that the diversification of intrinsic motoneuron electrical properties we observed during development might be dependent on a feedback signal from muscle fibers. To test this hypothesis, we injected botulinum toxin type A (BTX), which causes muscle paralysis (Thesleff...
et al. 1990; Watson 1969), into the left hind limb extensor muscles of young mice pups (P2-3),
and then measured the intrinsic motoneuron electrical properties of the treated motoneurons after
weight-bearing locomotion would have started at P8-11. We found that there were a number of
statistically significant differences in the intrinsic properties of motoneurons innervating
untreated control and botulinum-toxin treated extensor muscles. Portions of these data have been
published in abstract form (Nakanishi and Whelan 2008).

Materials and Methods

Experiments were performed on neonatal Swiss Webster mice (Charles River
Laboratories) 0-11 days old (n = 54 mice). The mice were anaesthetized by hypothermia (age ≤
P3) or by halothane (> P3) using procedures approved by the University of Calgary Animal Care
Committee.

Identification of motoneurons

In a preliminary set of experiments, 2.5% Fluorogold (FG, Fluorochrome, LLC, Denver,
CO, USA) was injected into the left hind limb ankle extensor muscle area using a 30 gauge
needle attached to a Hamilton syringe (Hamilton Company, Reno, NV, USA) at P0-1, then the
mice were sacrificed and slices prepared on P4 (Han et al. 2007). FG-positive, retrograde-
labeled motoneurons were consistently localized to the lateral ventral horn in lumbar spinal cord
segments 4-6, with somata diameter greater than ~20 µm. For subsequent experiments
examining intrinsic properties in mixed flexor and extensor motoneurons, motoneuron soma
from lumbar spinal cord segments 4-6 were visually identified (Olympus BX51WI) using infra-
red differential interference contrast (IR-DIC) in the lateral ventral horn and had soma diameters
greater than ~20 μm.

Botulinum toxin and Fluorogold Injections

In a subset of experiments (n=12), weight bearing, extensor muscle activity was blocked
with an intramuscular injection of botulinum toxin type A (BTX, Allergan Inc., Markham, OT,
Canada) into the triceps surae muscles. Postnatal mice (P2-3) were anesthetized by hypothermia,
then under aseptic conditions, 2-3 μl of a sterile saline solution (0.9% NaCl, Baxter Corporation,
Toronto, OT, Canada) containing 0.25 U BTX/μl with bovine serum albumin (1 μg/μl), and
2.5% FG (FG alone for control animals) was injected into the left hind limb (Fig. 5 A1). After
injections, the mice were placed on a heating pad to recover for 1-2 hours and then returned to
their home cage with their mother and BTX/FG-injected littermates. Injected mice were
examined daily for grooming and onset of muscle paralysis. Muscle paralysis was confirmed
either by electromyogram recording (EMG, details below, Fig. 5 A2) and/or the complete
absence of left hind limb movement during locomotion (supplementary video) and tail-
suspension. In a subset of experiments, FG alone, without BTX, was dissolved in sterile saline
and injected into hind limb extensor muscles to retrogradely label extensor motoneurons. These
FG-labeled extensor motoneurons, recorded at either ages P0-3 or P8-11, served as the control
data to compare against the BTX/FG-treated motoneurons (Summary Table 2).
Electromyogram Recording

In select preparations, we examined the EMG signals from control and BTX-treated hind limb muscles. The animals were anesthetized with halothane and two fine wire electrodes (75 μm; A-M Systems, Carlsborg, WA, USA) were inserted through the skin into the triceps surae muscle group. A ground wire was placed into the back of the animals. The animals were allowed to recover and movements were elicited by pinching the tail or the paw of each hind limb. No movements were observed from the treated hind limb. The EMGs were band-pass filtered (30 Hz-10 kHz), amplified (200 X) and recorded (AxoScope & Digidata 1322A, Molecular Devices) for offline analysis.

Tissue preparation

The procedure for tissue preparation has been documented in detail in a previous publication from our lab (Han et al. 2007). Briefly, the spinal cord was dissected free in ice-cold, sucrose-artificial cerebrospinal fluid (aCSF) solution bubbled with 95% O2-5% CO2 (concentrations in mM: 25 NaCl, 188 sucrose, 1.9 KCl, 10 MgSO4, 1.2 Na2HPO4, 26 NaHCO3, 25 D-glucose), and immediately transferred to a pre-cooled (4°C) slicing chamber and stabilized in an upright position onto an agar block using 20% gelatin. Transverse sections (250-300 μm) were cut (Leica Vibrotome VT1000S), and the slices were collected in a chamber containing pre-warmed (36°C), oxygenated recovery aCSF (concentrations in mM: 119 NaCl, 1.9 KCl, 1 CaCl2, 10 MgSO4, 1.2 Na2HPO4, 26 NaHCO3, 10 D-glucose), and equilibrated for at least 45 minutes before being placed into the recording chamber for IR-DIC visually-guided patch-clamp recordings.
Whole-cell patch-clamp

The lumbar spinal cord slice was placed into the recording chamber and superfused with oxygenated aCSF solution. The external oxygenated aCSF solution contained (in mM): 128 NaCl, 4 KCl, 1.5 CaCl₂, 1 MgSO₄, 0.5 Na₂HPO₄, 21 NaHCO₃, and 30 D-glucose. The internal pipette solution contained (in mM): 130 K Gluconate, 0.1 EGTA, 10 HEPES, 7 NaCl, 0.3 MgCl₂, ~0.4 KOH (pH to 7.3), 5 Di-Tris-Creatine, 2 ATP (4.8 Tris), 0.5 GTP (1.45 Na⁺).

Electrodes were pulled from borosilicate glass on a P97 Flaming/Brown puller (Sutter Instrument, Novato, CA) and had resistances in the range of 4-6 MΩ. The liquid junction potential between internal and external solutions was calculated using pClamp software (Molecular Devices, Sunnyvale, CA, USA) to be 11.6 mV and corrected. For the BTX/FG preparations, treated motoneurons were identified by the presence of FG-positive somata visualized by UV fluorescence. The data were low pass filtered (10 kHz) and digitized (sampling rate: 20 kHz) for off-line analyses (Digidata 1440A, Clampex and Clampfit 10, Molecular Devices, Sunnyvale, CA, USA).

Intrinsic Motoneuron Properties

A collection of intrinsic electrical properties was recorded from each motoneuron (total n= 140) in this study. These properties were: action potential amplitude, action potential half-width time, action potential time-to-peak, action potential threshold voltage, membrane capacitance, rheobase current, AHP half-decay time, input resistance, and frequency-current slope (fI gain). Action potential amplitude was measured from the resting potential preceding an action potential to that action potential peak. Action potential half-width time was measured as the duration of the spike at one-half the action potential amplitude (Fig. 1 A1). Action
potential time-to-peak time was measured from the base of an action potential to the peak.

Rheobase current was quantified as the minimal depolarizing current step (2 Hz, 25 ms duration, 5 pA intervals) sufficient to elicit an action potential (Fig. 2A). Averaged action potential traces (20 consecutive sweeps) evoked by brief supra-threshold depolarizing current injections (0.5-1.0 ms) were used to quantify action potential AHP half-decay times (AHP). AHP was measured as the duration from the most hyperpolarized potential following an action potential to the time at which the membrane potential has returned halfway to the resting potential. AHP duration was calculated from the downstroke of the action potential to the return to baseline. Input resistance ($R_{\text{in}}$) was measured by dividing the average voltage deflection (20 consecutive sweeps) of the membrane potential by a hyperpolarizing 50 pA current step (250 ms); and input conductance ($G_{\text{in}}$) was calculated from the $R_{\text{in}}$ measurement; $G_{\text{in}}=1/ R_{\text{in}}$. Whole cell membrane capacitance ($C_m$, pF) was recorded using the automated membrane test function in Clampex10 (Molecular Devices, Sunnyvale, CA, USA); briefly, in voltage clamp mode, after achieving the whole-cell configuration, a 10 mV command pulse is delivered and the resulting estimated integral of the current transient relative to the steady-state current during the pulse plus a steady-state correction factor are used to calculate the whole cell capacitance (pClamp 10 User guide, Rev. A).

Frequency-current slope (fI gain) was quantified by linear regression (all $r^2 >0.95$) to estimate the slope of the steady-state action potential firing rate in response to various amplitudes of depolarizing current steps (100pA steps, 2s).

**Data analyses**

Data analyses were performed using Clampfit (Molecular Devices, Sunnyvale, CA, USA). Data were evaluated using GraphPad Software (La Jolla, CA, USA). Treatment and age
effects were tested using Student’s t-tests or ANOVAs with Tukey post hoc tests with significance set at p<0.05, and non-significant (NS) p values are reported. Linear regression analysis was used to examine age-related effects, and 95% confidence intervals were also plotted. Coefficient of variations for the younger (P0-3) and older (P8-11) age groups were calculated for each day and then compared between the younger and older age groups (with the exception of the input resistance coefficient of variation in which days 10 and 11 were pooled because of small sample sizes (n=3) on each of those days), and reported as a percentage (%). Values reported as mean ± SEM unless otherwise noted.

Results

Normal developmental changes of intrinsic motoneuron electrical properties

The early postnatal period is a time of profound changes in the motor system, culminating in the onset of weight-bearing locomotion. Among the collection of central (Allain et al. 2005; Ballion et al. 2002; Clarac et al. 2004; Clarac et al. 1998; Gerin et al. 1995; Vinay et al. 2000b) and peripheral (Vullhorst et al. 1998; Wigston and English 1992; Yang et al. 1998) developmental changes that occur during the time between non weight-bearing and weight-bearing locomotion, we hypothesized that there would be significant changes in intrinsic motoneuron electrical properties. The following results, comparing the intrinsic electrical properties of motoneurons from a younger (P0-3) and older (P8-11) age group, were collected from combined flexor and extensor motoneurons in the lumbar spinal cord.

Action potential characteristics during early postnatal development
Upon achieving whole cell current clamp configuration, the membrane potential was set to ~ -70 mV by injecting a bias current, ranging from +50 to -300 pA. At this voltage, there were no spontaneous action potentials observed, and all intrinsic motoneuron properties were recorded starting with the motoneuron resting potential held at -70 mV. There was no statistically significant difference in the mean membrane capacitance, which is indicative of cell size, between the younger (P0-3: 88.73 ± 5.31 pF) and older age groups (P8-11: 99.50 ± 8.35 pF, p=0.27, NS), in a subset of motoneurons where Cm was measured. This suggests that differences in intrinsic motoneuron electrical properties described below are not due exclusively to changes in motoneuron cell size, though developmental changes in mouse motoneuron soma size and dendritic structure have been described (Li et al. 2005). Once the motoneuron’s resting membrane potential had stabilized over 2 to 4 minutes, small depolarizing square wave current steps (250 ms) of increasing amplitude were delivered until action potentials were elicited.

Action potential amplitude was measured from the first deflection point following the current step onset to the peak (Fig. 1 A1). Only motoneurons that exhibited action potentials over 40 mV were included for further study. The proportion of motoneurons that did not meet this criterion was evenly distributed across ages, was less than 10%, and no further data were recorded from these motoneurons. Generally, one motoneuron was recorded per slice, and up to 4-5 motoneurons were recorded from each mouse. Across the age range studied, from P0 to P11, there was no correlation between age and action potential amplitude (r²=0, p=0.94, Fig 1. A2). Likewise, when we directly compared the two targeted age groups, younger (P0-3) and older (P8-11), there was no statistically significant difference in mean action potential amplitude (younger: 74.06 ± 9.81 mV, older: 74.90 ± 14.80 mV, p=0.73).
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On the other hand, there was a statistically significant change in action potential half-width with respect to age. Action potential half-width was measured as the time between the upward and downward voltage deflections at the V_m of half the full action potential amplitude (Fig. 1 A1 and A2). Action potential half-width is dependent on a combination of factors; in spinal motoneurons, mainly changes in voltage-gated Na^+ channels and voltage-gated K^+ channels (Gao and Ziskind-Conhaim 1998), and potentially other conductances and passive membrane properties (Rekling et al. 2000). During the time period examined in this study, action potential half-width decreased with increasing age (r^2=0.17, p<0.001, Fig 1 B1.). The mean action potential half-width of motoneurons was significantly shorter in the older age group (P8-11: 0.97 ± 0.07 ms, p<0.0001) compared to the younger group (P0-3: 1.33 ± 0.05 ms).

There was also a statistically significant negative correlation between age and action potential time-to-peak (TTP), measured as the time between the action potential onset to the action potential peak (r^2= 0.05, p<0.05, Fig 1 A1 and B2), and accordingly, there was a significant difference in TTP between the younger (P0-3: 0.98 ± 0.04 ms) and older age groups (P8-11: 0.81 ± 0.06, p<0.05). There was no significant correlation of age and voltage threshold (r^2=0.009, p=0.34, NS; Fig. 1 C1 and C2), but a significant difference in action potential threshold voltage between the younger (-51.86 ± 0.77 mV) and older age groups (-56.00 ± 2.12 mV, p<0.05), a trend consistent with previous results showing a more hyperpolarized I_{Na} threshold as postnatal development progresses in rat motoneurons (Gao and Ziskind-Conhaim 1998). Taken together, these results demonstrate that the collection of motoneuron properties that mediate Na^+ spikes change during early postnatal development (Gao and Ziskind-Conhaim 1998).
Developmental changes in rheobase current and rheobase current diversity

Orderly recruitment of motoneurons is dependent in part on the diversity of excitability of motoneurons (Burke 1981; Cope and Pinter 1995; Desmedt and Godaux 1981; Kernell 2006). The mean rheobase current increased significantly during the early postnatal period examined in this study. A comparison of the targeted age groups showed that the mean rheobase current was significantly greater in the older age group (P8-11: 287.70 ± 40.71 pA) compared to the younger age group (P0-3: 157.60 ± 8.41 pA, p<0.0001, Fig. 2C). The age-related increase in mean rheobase current was attained in the older age group by a significant increase in the overall mean along with the range of rheobase currents measured (Fig. 2B). In the older age group, some motoneurons exhibited rheobase current values within the range of the younger age group, while a subset of motoneurons expanded beyond this range. Some rheobase current measures of motoneurons from the older age group were up to 5 times greater than the largest rheobase current measures of motoneurons from the younger age group. One interpretation of this change in the range of rheobase current measures is that it represents an increase in diversity. To test whether this increase in diversity was statistically significant, we measured the coefficient of variation of rheobase current measures for each day in each age group, and then compared the mean coefficients of variation. We found that there was a significant increase in the coefficient of variation (CV) in the older age group (79.42%) compared to the younger age group (38.15%, p<0.05, Fig. 2C).

Developmental changes in AHP and diversity

In motoneurons, the AHP has been divided into two distinct processes, a fast phase (2-10 ms (Nordstrom et al. 2007), and a slow phase also termed a medium AHP (Powers and Binder
A third very slow phase (Sah 1996) found in some neurons has not been observed in motoneuron AHPs thus far (Powers and Binder 2001; Stauffer et al. 2007). In this study we examined the medium, apamin-sensitive AHP that contributes to action potential firing rate regulation during repetitive firing (Fig. 3 A). The AHP has two attributes, amplitude and time course; a composite measure of these two attributes is the AHP half-decay time that was quantified as the time between the downward and upward voltage deflections at half the AHP amplitude.

In the present study, twenty consecutive AHP responses to a brief supra-threshold current injection (0.5 ms, 2-3 nA) were recorded, then the AHP half-decay time was calculated as described above. There was no statistically significant difference in AHP half-decay time between the older and younger age groups (younger: 45.31 ± 10.83 ms, older: 48.63 ± 20.15 ms, p=0.30, Fig 3B and 3C). Although there was no significant difference in the mean AHP half-decay time, there was a significant increase in the coefficient of variation between the younger (22.07%) and older age groups (43.36%, p<0.001). This result suggests that although there was no change in the mean AHP half-decay time, there was an age-correlated increase in the diversity of AHP half-decay time not unlike the increase in diversity of rheobase current (see above). After-depolarizations that are a feature of developing motoneurons (Navarrete and Vrbova 1993) were consistently observed at all age points examined.

Developmental changes in input resistance diversity

Input resistance ($R_{in}$) was recorded by delivering small hyperpolarizing current steps (-50 pA, 250 ms at 0.5 Hz, 10 sweeps averaged) and the resulting voltage deflecting in membrane
potential was also recorded. There was no statistically significant difference in the mean input resistance measures between the younger (99.47 ± 6.45 MΩ) and older age groups (87.27 ± 10.23 MΩ, p=0.30, Fig. 4 A1 and A2). However, there was a significant difference in input resistance in the coefficient of variation between the age groups (younger: 41.29%, older: 79.81%, p<0.05) that is consistent with an increase in the coefficient of variation of motoneuron excitability (Fig. 4 A2).

Previous studies have noted a significant positive relationship between rheobase current and input conductance (G_{in}=1/R_{in}) in motoneurons recorded from young rats (Seebach and Mendell 1996). Examining the data presented in the current study using linear regression showed a rather weak relationship (r^2=0.28) between rheobase current and input conductance in the younger age group (P0-3), and this relationship became somewhat stronger in the older age group (P8-11, r^2=0.55). Linear regression analysis further showed that the relationship between rheobase current and input conductance in both age groups had slopes significantly different from zero (P0-3, p<0.01; P8-11, p<0.01), but similar to Seebach and Mendell, these slopes were not significantly different from each other (F=0.003, p=0.96).

No significant changes in fI gain during neonatal development

Average action potential firing rates were recorded in response to multiple supra-threshold, 2 second depolarizing current steps of varying amplitudes. Steady-state firing rates were calculated as the average action potential firing rate of the last 7 action potentials recorded for each current step (Fig. 4 B1). The slope of the average firing rate in response to various current steps is reported as fI gain. There was no statistically significant difference in the mean.
fl gains (younger: 56.26 ± 3.03 Hz/nA, older: 56.09 ± 8.55 Hz/nA, p=0.98, Fig. 4 B2) or the associated coefficients of variation (younger: 32.03%, older: 33.59%, p=0.92, Fig. 4 B2) between the younger and older age groups.

All intrinsic property values for combined flexor and extensor motoneurons recorded from the younger (P0-3) and older (P8-11) age groups are reported in Summary Table 1.

Botulinum toxin-induced muscle paralysis and the development of intrinsic motoneuron electrical properties

During postnatal development, motoneurons first form neuromuscular junctions (NMJs) with potential target muscle fibers prenatally between embryonic day 12 to 14 in mice (Jansen and Fladby 1990). A number of studies have investigated various aspects of the process of neuromuscular junction formation and these studies have collectively illuminated mechanisms regarding trophic processes (Thesleff et al. 1990), apoptosis (Carr and Simpson 1978), cell-cell signaling, synapse formation (Sanes and Lichtman 1999), activity-dependent synaptic plasticity and homeostasis (Wang et al. 2006; Wang et al. 2005). Although many of these processes are initiated during embryonic development, many of them continue through adulthood. More specifically, in terms of intrinsic motoneuron electrical properties, recent studies on adult mammals have shown that the maintenance of intrinsic motoneuron electrical properties and their diversity is critically dependent on interactions with muscle fibers (Nakanishi et al. 2005; Pinter et al. 1991). We wondered if BTX-induced paralysis of extensor muscles, which are critical for weight-bearing locomotion, would alter the developmental diversification of extensor motoneuron electrical properties during early postnatal development.
To test the hypothesis that BTX-induced muscle paralysis would interfere with the normal diversification of intrinsic motoneuron electrical properties, we injected BTX/FG or control, FG-only into the left hind limb of postnatal mice (P0-2, Fig. 5 A1) before they began weight-bearing locomotion (Brown et al. 1982). The solution we injected into the extensor muscles included Fluorogold (FG), which retrogradely-labeled the motoneurons that innervate the extensor muscles (also see methods, above). Then either 1-2 or 7-9 days later, spinal cord slices were prepared and FG-positive motoneurons were identified and their intrinsic properties were recorded as described above. In a subset of experiments, EMGs were recorded from BTX-treated and control hind limb muscles, and we found that we could evoke no observable EMG signals in the BTX-treated hind limbs (Fig. 5 A2).

**Action potential characteristics following BTX-induced muscle paralysis**

There were no significant differences in action potential amplitude (control P0-3: 79.67 ± 1.98 mV, control P8-11: 72.62 ± 3.63 mV, BTX P8-11: 71.57 ± 2.23, ANOVA, p=0.06, NS, Fig. 6 A2), or action potential threshold voltage (control P0-3: -51.67 ± 1.94 mV, control P8-11: -54.41 ± 2.16 mV, BTX P8-11: -58.09 ± 2.01 mV, ANOVA, p=0.08, NS, Fig. 6 A5), and there was no statistically significant difference in membrane capacitance between control P0-3 (86.64 ± 7.271 pF), control P8-11 (62.63 ± 4.243 pF) and BTX P8-11 (74.86 ± 11.61 pF) extensor motoneurons (ANOVA, p=0.06, NS).

**Development of rheobase current diversity during muscle paralysis**
In this present study, we hypothesized that BTX-induced muscle paralysis would significantly alter the mean rheobase current that would accompany normal development (see above). BTX-induced muscle paralysis led to a statistically significant increase in rheobase current compared to untreated, control extensor motoneurons. The mean rheobase current of extensor motoneurons after BTX treatment (P8-11, 252.40 ± 46.31 pA) was significantly different from that of motoneurons from control animals at both P0-3 (133.50 ± 14.54 pA) and P8-11 (139.00 ± 20.54, ANOVA, p<0.05, Fig. 7 A), but there was no statistically significant difference in the coefficient of variation of rheobase current (control P0-3: 54.55%, control P8-11: 59.18%, and BTX P8-11: 72.98%, Fig. 7A). These results demonstrate that BTX-induced extensor muscle paralysis led to a significant decrease in the excitability (i.e. increase in rheobase current) of extensor motoneurons.

Effects of muscle paralysis on the AHP

BTX-induced muscle paralysis during early postnatal development had no statistically significant effect on AHP half-decay time. Motoneurons that innervated BTX-treated muscles had mean AHP half-decay time measures (control P0-3: 40.77 ± 4.94 ms, control P8-11: 48.32 ± 4.96 ms, BTX P8-11: 39.39 ± 4.30 ms, ANOVA, p=0.43, NS, Fig. 7B) and coefficients of variation (control P0-3: 39.33%, control P8-11: 36.66%, BTX P8-11: 47.11%, ANOVA, p=0.78) that were statistically indistinguishable from motoneurons from either control age group (Fig. 7B).

Input resistance and fI gain development during muscle paralysis
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BTX-induced muscle paralysis led to a statistically significant change in mean input resistance. Motoneurons that had innervated BTX-treated muscles had a mean input resistance that was significantly lower than control extensor motoneurons in both age groups (control P0-3: 158.30 ± 27.41 MΩ, control P8-11: 156.20 ± 26.89, BTX P8-11: 72.31 ± 9.46, ANOVA, p<0.05, Fig. 8A), while there was no significant difference in input resistance coefficient of variation (control P0-3: 77.41%, control P8-11: 69.52%, BTX P8-11: 54.04%, ANOVA, p=0.50, NS, Fig. 8A). Following BTX treatment, extensor motoneuron fl gains from treated and controls had statistically indistinguishable fl gains. There were no significant differences in the mean fl gains (control P0-3: 57.41 ± 7.16 Hz/nA, control P8-11: 71.17 ± 9.41 Hz/nA, BTX P8-11: 63.91 ± 13.31 Hz/nA, ANOVA, p=0.54, NS, Fig. 8B) of the fl gain coefficient of variation (control P0-3: 45.34%, control P8-11: 39.49%, BTX P8-11: 47.47%, ANOVA, p=0.78, NS, Fig. 8B).

The intrinsic property values for control (P0-3 & P8-11) and BTX-treated (P8-11) extensor motoneurons are reported in Summary Table 2.

Discussion

In this manuscript we demonstrate that motoneuron-activated vesicle release, and the resulting muscle activity, leads to sculpting of intrinsic motoneuronal properties in neonatal mice. We show that paralysis of extensor muscles induced by BTX injections leads to a decrease in the excitability of extensor motoneurons. Interestingly, this dynamic remodeling of intrinsic properties occurs during a time when both slow and fast motoneurons exhibit phasic firing properties (Navarrette and Vrbova 1993). We further show that under normal conditions there is
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a gradual increase in the diversity of the motoneuronal pool in mice during the first days following birth with both similarities and differences to the neonatal rat.

The correlation between the developmental changes in intrinsic motoneuron electrical properties and the onset of weight-bearing locomotion suggests three possible interpretations: one, developmental diversification of motoneuron properties directly contributes to weight-bearing locomotion; two, weight-bearing locomotion (and the associated action potential initiated muscle contractions, muscle activity) initiates mechanisms leading to motoneuron diversification; or three, that weight-bearing locomotion and motoneuron diversification are temporally correlated, but are not causally related. A survey of changes in intrinsic motoneuron electrical properties before and after the onset of weight-bearing locomotion alone cannot distinguish among these possibilities. So we tested the hypothesis that precluding weight-bearing locomotion by paralyzing hind limb extensor muscles with intra-muscular BTX injections would affect the development and diversification of intrinsic motoneuron electrical properties. In our work, we observed that in extensor MNs inactivity led to an increase in the rheobase current. In adult rats TTX induced paralysis also increased the rheobase but only in presumptive slow type (S) motoneurons with long duration AHPs (Cormery et al. 2000); this effect was generalizable to extensor motoneurons in which muscles were unloaded by tail suspension (Cormery et al. 2005). Changes in the opposite direction were observed (decrease in rheobase, increase in input resistance) when the medial gastrocnemius nerve was chronically stimulated (Munson et al. 1997). Beaumont and Gardiner (2002) found that running in rats showed a shift in the voltage threshold towards more negative values (although the resting membrane potential was also reduced). Thus our results fit with the general idea that activity
promotes MN excitability. In adults, BTX application generally mimics the effects of axotomy that is to increase excitability by reducing the rheobase current and increasing the input resistance (Foehring and Munson 1990; Nakanishi et al. 2005; Pinter et al. 1991). That said, the effects observed with BTX are smaller than that observed following axotomy, likely due to the fact that not all neuromuscular transmission is blocked by BTX (Nakanishi et al. 2005). Interestingly, we observed the opposite effect during early development whereby BTX led to an increase in the rheobase and a decrease in the Rin. It appears that BTX at young ages tends to mimic the effects of other strategies used to block muscle activity (TTX & loading strategies). A contributing issue is that blocking muscle activity in neonates prolongs the time during which muscle fibers are poly-neuronally innervated (Benoit and Changeux 1978; 1975; Caldwell and Ridge 1983; Thompson et al. 1984). Furthermore, axotomy leads to motoneuronal cell death in neonates which does not occur in adults (Greensmith et al. 1997). Taken together, it appears that there are factors in neonates that can influence activity-dependent sculpting of motoneuronal properties. A likely candidate is a retrograde messenger from the muscle fiber to the motoneuron as has been postulated for adult animals (Nakanishi et al. 2005; Pinter et al. 1991). If this is the case, our data suggests that this type of messenger molecule is active early in neonatal development.

Comparison of mouse and rat motoneuron development

The present study on mice supports and contrasts with findings from a previous study by Fulton and Walton (1986) that described developmental changes in rat lumbar motoneuron intrinsic electrical properties during postnatal development (P3-12, n=44 motoneurons). In the study by Fulton and Walton, a table is presented showing motoneurons and their measured
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- Electrical properties at various ages, and using the data in that table, we analyzed their data for age-associated changes and compared their results with ours. In both our study and Fulton and Walton’s, there was no significant correlation between age and action potential amplitude or AHP times. The AHP durations we observed in neonates were substantially longer than those seen in adult mice (Manuel et al. 2009) similar to the data obtained from rats. In both studies, there was a significant negative correlation between age and action potential half-width (our study: slope = -0.05 ms/day, Fulton and Walton: slope = -0.05 ms/day, $r^2=0.12$, $p<0.05$). In contrast, Fulton and Walton’s data show a significant negative correlation between age and input resistance ($r^2=0.45$, $p<0.0001$) while we found no statistically significant relationship ($r^2=0.03$, $p=0.06$, NS). However, we did observe that the range of values recorded significantly increased. In other words, we recorded from more motoneurons with lower input resistances as the age increased. However, we also found motoneurons with higher input resistances, thus although a trend for a decrease was present, it was not statistically significant. Finally, the Fulton and Walton study provide some data (n=10 motoneurons) on current injection and action potential firing (i.e. rheobase current), though some of their current injection amplitudes are insufficient for repetitive firing which is probably greater than the minimum current sufficient to evoke a single action potential. Dividing their data into two age groups, younger (P3-5, n=5) and older (P6-11, n=5), there was no significant difference in their rheobase current measures between the two age groups ($p=0.86$), which contrasts with our results, though the rheobase CV measures of the Fulton and Walton data are similar (Fulton and Walton: 35.57 and 84.27%; our study: 38.15 and 79.42%; younger and older, respectively). In a separate study on developing rat motoneurons, Seebach and Mendell found a significant increase in rheobase current between younger (P1-3: $1.0 \pm 0.7$ (SD) nA) and older (P7-9: $2.9 \pm 1.7$ (SD) nA), with an accompanying increase in
standard deviation (Seebach and Mendell 1996). Taken together, these imperfect comparisons suggest that there may be differences in the time course and/or postnatal development of intrinsic motoneuron electrical properties between rats and mice.

Specialization of extensor motoneurons

The ankle-extensor triceps surae muscles in mice contain a mixture of slow and fast motoneurons. However, the ratio of fast to slow muscle fibers is reduced compared to the tibialis anterior and extensor digitorum longus muscles. At birth, twitch speed tends to be similarly slow for both fast and slow muscles. During the first neonatal week both slow and fast muscles speed up at a similar rate (Close 1964). This is followed by a secondary slowing of slow muscles in soleus for example accompanied by an increase in the percentage of slow type I fibers (Kugelberg, 1976). In our work, differences between a pure extensor group and a mixed flexor and extensor group emerged only at later stages of neonatal development (P8-11). Specifically, the extensor group was more excitable than the mixed group, possibly due to a higher percentage of slow fibers in anti-gravity muscles (Burkholder et al. 1994). This confirms data from rats in which significant differences were observed between extensor and flexor motoneuron intrinsic properties (Vinay et al. 2000a). The interpretation of our data is that before specialization of muscle fiber types into functional fast and slow twitch times, extensor motoneuron properties can be modified by activity associated with muscle contraction. It must be pointed out that changes in the AHP are generally associated with activity-dependent modification of motoneuronal properties, and it appears this is especially true for primarily slow muscles (Cormery et al. 2000).

In our work, we observed no significant change in the AHP between BTX and control groups, nor in fact did we observe any differences over time. This matches with data from the rat where
motoneurons keep their rather long AHP over the first two weeks (Fulton and Walton 1986). Therefore it would appear that while activity appears to be capable of modifying the rheobase and input resistance during young ages, the AHP remains unaffected; or perhaps AHP changes occur over a notably longer time course. Clearly, at some point during development from P14 to adulthood, the effects of activity on intrinsic properties shift, since it known that activity can modify muscle properties and AHPs within presumptive slow motoneurons (Cormery et al. 2000; Munson et al. 1997). It would appear that this shift coincides with the maturation of anti-gravity muscles, as they carry more load as the animal matures.

Conclusions

Our work demonstrates that activity plays an important role in the development of motoneuronal intrinsic properties. In particular, we observed that paralysis reduces the excitability of motoneurons. Therefore, normal extensor activity during postnatal life appears to be crucial even though activity levels are lower as a consequence of limited mobility along with slow motor units responding phasically rather than tonically. An important question is whether these changes in extensor motoneuron intrinsic properties are determined by changes in a retrograde signaling factor or by changes in synaptic drive arising from muscle afferents. Future work will be required to dissect these possibilities.

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Grants

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Figure Legends

**Figure 1.** Action potential characteristics of motoneurons recorded at various postnatal ages.

*A1,* Examples of action potentials recorded from motoneurons at P1 (grey) and P8 (black); action potential amplitude (solid line) and action potential half-width (dashed line between arrowheads).  
*A2,* Action potential amplitudes showed no significant correlation with age.  
*B1,* Action potential half-width was significantly negatively correlated with age, as was action potential time-to peak.  
*C1,* Action potential voltage threshold example traces, P1 (grey) and P8 (black), (with truncated action potentials).  
*C2,* Action potential voltage threshold was not significantly correlated with age.

**Figure 2.** Mean rheobase current and rheobase current diversity both increase during postnatal development.  
*A,* Example of rheobase current measurement.  
*B,* Scatter plot showing rheobase current measurements from each motoneuron recorded at various postnatal ages.  
*C,* Comparisons of mean rheobase current (left) and rheobase current coefficient of variation (right) between younger (P0-3) and older (P8-11) age groups, error bars ± SEM.

**Figure 3.** AHP half-decay time during postnatal development.  
*A,* Example of averaged AHP with preceding action potential truncated.  
*B,* Plot shows AHP half-decay time measurements at various postnatal ages.  
*C,* Comparisons of mean AHP half-decay times between younger (P0-3) and older (P8-11) age groups and AHP half-decay time coefficients of variation between the two age groups, error bars ± SEM.
Figure 4. Input resistance and action potential frequency-current relationships. A1, Plot shows input resistance measurements from each motoneuron at various postnatal ages. A2, Comparisons of mean input resistance and input resistance coefficient of variation between the younger (P0-3) and older (P8-11) age groups. B1, Examples of repetitive action potential firing in response of two levels of current injection. B2, Comparisons of action potential frequency-current relationships (fI gains) and fI gain coefficients of variations of younger (P0-3) and older age groups (P8-11), error bars ± SEM.

Figure 5. Botulinum toxin-induced muscle paralysis and action potential characteristics following BTX-induced muscle paralysis. A1, Diagram of BTX injection location. A2, Electromyogram recordings (details in methods) from untreated, control (top) and BTX-treated hindlimb muscles after BTX injections (bottom).

Figure 6. Comparisons of action potential characteristics between motoneurons from control (P0-3) control (P8-11) and BTX-treated (P8-11) mice, error bars ± SEM. A1, Examples of action potentials recorded from extensor motoneurons: control P0-3 (dashed line), control P8-11 (solid black line) and BTX-treated extensor motoneuron (grey). A2, Action potential amplitudes showed no statistically significant differences. A3, Comparison of action potential half-width (ms). A4, Comparison of action potential time-to-peak (ms) and statistically significant differences. A5, No statistically significant difference in action potential threshold between control (P0-3), control (P8-11) and BTX-treated (P8-11) extensor motoneurons.
Figure 7. Rheobase current and AHP half-decay time of control (P0-3), control (P8-11) and BTX-treated (P8-11) extensor motoneurons, error bars ± SEM. **A**, Comparison of mean rheobase current with statistically significant differences and coefficients of variation (CV) of rheobase current. **B**, Comparison of mean AHP and AHP coefficients of variation.

Figure 8. Input resistance and action potential frequency-current relationships of control (P0-3), control (P8-11) and BTX-treated (P8-11) extensor motoneurons, error bars ± SEM. **A**, Comparison of mean input resistance with statistically significant differences and input resistance coefficients of variation. **B**, Comparison of mean action potential frequency-current relationships (fI gains) and coefficients of variation of fI gains.

Tables

**Summary Table 1**: Developmental changes of combined flexor and extensor motoneuron intrinsic electrical properties.

**Summary Table 2**: Motoneuron electrical properties recorded from untreated, control extensor motoneurons (P0-3), control (P8-11) and botulinum toxin-treated P8-11 extensor motoneurons.
Figure 4

A1

A2

B1

B2

Input resistance (MΩ)

Age group (days)

Input resistance (MΩ)

Input resistance coefficient of variation (CV)

Age group (days)

fl gain coefficient of variation

Age group (days)

fl gain (Hz/nA)

Age group (days)
Figure 5

A1

A2

Control

0.50 U Botulinum toxin

3 s
Figure 6

A1

- Dotted line: P0-3
- Solid line: P8-11
- Dashed line: BTX

Scale: 10 mV, 2 ms

A2

Action potential amplitude (mV)

<table>
<thead>
<tr>
<th>Age and treatment group</th>
<th>Action potential amplitude (mV)</th>
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<tbody>
<tr>
<td>P0-3</td>
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<tr>
<td>P8-11</td>
<td>70</td>
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<td>BTX P8-11</td>
<td>60</td>
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A3

Action potential half-width (ms)

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<tr>
<td>P0-3</td>
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<tr>
<td>P8-11</td>
<td>1.45</td>
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<tr>
<td>BTX P8-11</td>
<td>1.40</td>
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A4

Action potential time-to-peak (ms)

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<tr>
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<tr>
<td>P8-11</td>
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</tr>
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<td>BTX P8-11</td>
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A5

Voltage threshold (mV)

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<td>P0-3</td>
<td>-50</td>
</tr>
<tr>
<td>P8-11</td>
<td>-55</td>
</tr>
<tr>
<td>BTX P8-11</td>
<td>-60</td>
</tr>
<tr>
<td>Number of motoneurons (n)</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>P0-3</td>
</tr>
<tr>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Action potential amplitude (mV)</td>
<td>74.06 ± 1.16</td>
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<tr>
<td>Action potential half-width (ms)</td>
<td>1.33 ± 0.05</td>
</tr>
<tr>
<td>Action potential time-to-peak (ms)</td>
<td>0.98 ± 0.04</td>
</tr>
<tr>
<td>Action potential threshold (mV)</td>
<td>-51.86 ± 0.77</td>
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<tr>
<td>Rheobase current (pA)</td>
<td>157.60 ± 8.41</td>
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<tr>
<td>Rheobase current CV</td>
<td>38.15%</td>
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<tr>
<td>After-hyperpolarization hd time (ms)</td>
<td>45.31 ± 10.83</td>
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<td>AHP hd CV</td>
<td>22.07%</td>
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<td>After-hyperpolarization duration (ms)</td>
<td>152.80 ± 5.13</td>
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<tr>
<td>Input resistance (MΩ)</td>
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<td>Input resistance CV</td>
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<tr>
<td>fi gain (Hz/nA)</td>
<td>56.26 ± 3.03</td>
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<tr>
<td>fi gain CV</td>
<td>32.03%</td>
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Summary Table 2. *Intrinsic electrical properties of control and BTX-treated extensor motoneurons*

<table>
<thead>
<tr>
<th>Number of motoneurons (n)</th>
<th>Control P0-3</th>
<th>Control P8-11</th>
<th>BTX P8-11</th>
<th>ANOVA</th>
<th>p value</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Action potential amplitude (mV)</td>
<td>79.67 1.98</td>
<td>72.62 3.63</td>
<td>71.57 2.23</td>
<td>NS</td>
<td>(0.06)</td>
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<tr>
<td>Action potential half-width (ms)</td>
<td>1.34 0.18</td>
<td>1.53 0.13</td>
<td>1.14 0.08</td>
<td>NS</td>
<td>(0.15)</td>
</tr>
<tr>
<td>Action potential time-to-peak (ms)</td>
<td>0.87 0.06</td>
<td>1.31 0.15</td>
<td>0.91 0.05</td>
<td>&lt;0.01</td>
<td>*</td>
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<tr>
<td>Action potential threshold (mV)</td>
<td>-51.67 1.94</td>
<td>-54.41 2.16</td>
<td>-58.09 2.01</td>
<td>NS</td>
<td>(0.08)</td>
</tr>
<tr>
<td>Rheobase current (pA)</td>
<td>133.50 14.54</td>
<td>139.00 20.54</td>
<td>252.40 46.31</td>
<td>&lt;0.05</td>
<td>*</td>
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<tr>
<td>Rheobase current CV</td>
<td>54.55%</td>
<td>59.18%</td>
<td>72.98%</td>
<td>NS</td>
<td>(0.57)</td>
</tr>
<tr>
<td>After-hyperpolarization hd time (ms)</td>
<td>40.77 4.94</td>
<td>48.32 4.96</td>
<td>39.39 4.30</td>
<td>NS</td>
<td>(0.43)</td>
</tr>
<tr>
<td>AHP hd CV</td>
<td>39.33%</td>
<td>36.66%</td>
<td>47.11%</td>
<td>NS</td>
<td>(0.78)</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>158.30 27.41</td>
<td>156.20 26.89</td>
<td>72.31 9.46</td>
<td>&lt;0.05</td>
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<tr>
<td>Input resistance CV</td>
<td>77.41%</td>
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<td>(0.50)</td>
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<td>fI gain (Hz/nA)</td>
<td>57.41 7.16</td>
<td>71.19 9.41</td>
<td>63.91 13.31</td>
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<td>(0.54)</td>
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<td>fI gain CV</td>
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<td>39.49%</td>
<td>47.47%</td>
<td>NS</td>
<td>(0.78)</td>
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