Diversification of Intrinsic Motoneuron Electrical Properties During Normal Development and Botulinum Toxin–Induced Muscle Paralysis in Early Postnatal Mice

S. T. Nakanishi and P. J. Whelan
Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada

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Nakanishi ST, Whelan PJ. Diversification of intrinsic motoneuron electrical properties during normal development and botulinum toxin–induced muscle paralysis in early postnatal mice. J Neurophysiol 103: 2833–2845, 2010. First published March 21, 2010; doi:10.1152/jn.00022.2010. 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) of rheobase current, afterhyperpolarization half-decay time, and input resistance. In various adult mammalian preparations, the maintenance of intrinsic motoneuron electrical properties is dependent on 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.

IN T R O D U C T I O N

During early postnatal development of the CNS, α-motoneurons in the spinal cord go through significant morphological and physiological changes (Fulton and Walton 1986; Li et al. 2005; Perrier and Houmsgaard 2000; Vinay et al. 2000b). Among the previously reported early postnatal changes in the intrinsic electrical properties of motoneurons are changes in input resistance ($R_{\text{in}}$) and afterhyperpolarization (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 nonweight-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–P3), and a second set after the onset of weight-bearing locomotion, P8–P11. 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 (AP) half-width, rheobase current (measure of motoneuron excitability), AHP half-decay time (contributes to firing rate regulation), and input resistance ($R_{\text{in}}$).

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 hindlimb extensor muscles of young mice pups (P2–P3) and then measured the intrinsic motoneuron electrical properties of the treated motoneurons after weight-bearing locomotion would have started at P8–P11. We found that there were a number of
statistically significant differences in the intrinsic properties of motoneurons innervating untreated control and BTX-treated extensor muscles. Portions of these data have been published in abstract form (Nakanishi and Whelan 2008).

METHODS

Experiments were performed on neonatal Swiss Webster mice (Charles River Laboratories) 0–11 days old (n = 54 mice). The mice were anesthetized 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, Denver, CO) was injected into the left hindlimb ankle extensor muscle area using a 30 gauge needle attached to a Hamilton syringe (Hamilton, Reno, NV) at P0–P1, after which the mice were killed 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 ≥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 infrared differential interference contrast (IR-DIC) in the lateral ventral horn and had soma diameters ≥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, Markham, Ontario, Canada) into the triceps surae muscles. Postnatal mice (P2–P3) were anesthetized by hypothermia; then, under aseptic conditions, 2–3 μl of a sterile saline solution (0.9% NaCl, Baxter, Toronto, Ontario, 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 hindlimb (Fig. 5A1). After injections, the mice were placed on a heating pad to recover for 1–2 h 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 in the following text, Fig. 5A2) and/or the complete absence of left hindlimb movement during locomotion (Supplemental Video) and tail suspension.1 In a subset of experiments, FG alone, without BTX, was dissolved in sterile saline and injected into hindlimb extensor muscles to retrogradely label extensor motoneurons. These FG-labeled extensor motoneurons, recorded at either ages P0–P3 or P8–P11, served as the control data to compare against the BTX/FG-treated motoneurons (see Table 2 in the following text).

Electromyogram recording

In select preparations, we examined the EMG signals from control and BTX-treated hindlimb muscles. The animals were anesthetized with halothane and two fine wire electrodes (75 μm; A-M Systems, Carlsborg, WA) 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 hindlimb. No movements were observed from the treated hindlimb. The EMGs were band-pass filtered (30 Hz to 10 kHz), amplified (×200), and recorded (Axoscope and Digidata 1322A; Molecular Devices, Sunnyvale, CA) for off-line 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 was immediately transferred to a precooled (4°C) slicing chamber and stabilized in an upright position onto an agar block using 20% gelatin. Transverse sections (250–300 μm) were cut (VT1000S; Leica Vibrotome); the slices were collected in a chamber containing prewarmed (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 ≥45 min 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-glucanolate 0.1 EGTA, 10 HEPES, 7 NaCl, 0.3 MgCl2, approximately 0.4 KOH (pH to 7.3), 5 di-Tris-creatine, 2 ATP (4.8 Tris), and 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) 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 ultraviolet 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).

Intrinsic motoneuron properties

A collection of intrinsic electrical properties was recorded from each motoneuron (total n = 140) in this study. These properties were: AP amplitude, AP half-width time, AP time-to-peak, AP threshold voltage, membrane capacitance (Cm), rheobase current, AHP half-decay time, Rm and frequency–current slope (f–I gain). AP amplitude was measured from the resting potential preceding an AP to that AP peak; AP half-width time was measured as the duration of the spike at 0.5 the AP amplitude (Fig. 1A1); AP time-to-peak was measured from the base of an AP 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 AP (Fig. 2A). Averaged AP traces (20 consecutive sweeps) evoked by brief suprathreshold depolarizing current injections (0.5–1.0 ms) were used to quantify AP AHP half-decay times (AHP). AHP was measured as the duration from the most hyperpolarized potential following an AP to the time at which the membrane potential has returned halfway to the resting potential. AHP duration was calculated from the downstroke of the AP to the return to baseline. Input resistance (Rm) 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 (Gm) was calculated from the Rm measurement: Gm = 1/Rm. Whole cell membrane capacitance (Cm, pF) was recorded using the automated membrane test function in Clampex10 (Molecular Devices); 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

1 The online version of this article contains supplemental data.
Data analyses

Data analyses were performed using Clampfit (Molecular Devices). Data were evaluated using GraphPad Software (La Jolla, CA). Treatment and age effects were tested using Student’s t-test or ANOVAs with Tukey post hoc tests, with significance set at $P < 0.05$, and nonsignificant (NS) $P$ values are reported. Linear regression analysis was used to examine age-related effects and 95% confidence intervals were also plotted. Coefficients of variation (CVs) for the younger (P0–P3) and older (P8–P11) age groups were calculated for each day and then compared between the younger and older age groups [with the exception of the $R_{\text{in}}$ CV, 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 are reported as means ± SE 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. 1998, 2004; 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 nonweight-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 the younger (P0–P3) and older (P8–P11) age groups, were collected from combined flexor and extensor motoneurons in the lumbar spinal cord.

**Action potential characteristics during early postnatal development**

On achieving whole cell current clamp configuration, the membrane potential was set to approximately −70 mV by injecting a bias current, ranging from +50 to −300 pA. At this voltage, no spontaneous APs were 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–P3: 88.73 ± 5.31 pF) and older age groups (P8–P11: 99.50 ± 8.35 pF, $P = 0.27$, NS), in a subset of motoneurons in which $C_m$ was measured. This suggests that differences in intrinsic motoneuron electrical properties described in the following text are not due exclusively to changes in motoneuron cell size, although 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 min, small depolarizing square wave current steps (250 ms) of increasing amplitude were delivered until APs were elicited.

Action potential amplitude was measured from the first deflection point following the current step onset to the peak (Fig. 1A1). Only motoneurons that exhibited APs >40 mV were included for further study. The proportion of motoneurons that did not meet this criterion was evenly distributed across ages, was <10%, and no further data were recorded from these motoneurons. Generally, one motoneuron was recorded per slice and up to four to five motoneurons were recorded from each mouse. Across the age range studied, from P0 to P11, there was no correlation between age and AP amplitude ($r^2 = 0$, $P = 0.94$, Fig. 1A2). Likewise, when we directly compared the two targeted age groups, younger (P0–P3) and older (P8–P11), there was no statistically significant difference in mean AP amplitude (younger: 74.06 ± 9.81 mV; older: 74.90 ± 14.80 mV, $P = 0.73$).
On the other hand, there was a statistically significant change in AP 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 AP 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, AP half-width decreased with increasing age ($r^2 = 0.17$, $P < 0.001$, Fig. 1BI). The mean AP half-width of motoneurons was significantly shorter in the older age group (P8–P11: 0.97 ± 0.07 ms, $P < 0.0001$), compared with that of the younger group (P0–P3: 1.33 ± 0.05 ms). There was also a statistically significant negative correlation between age and AP time-to-peak (TTP), measured as the time between the AP onset to the AP peak ($r^2 = 0.05$, $P < 0.05$, Fig. 1, A1 and B2); accordingly, there was a significant difference in TTP between the younger (P0–P3: 0.98 ± 0.04 ms) and older age groups (P8–P11: 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, CI and C2), but a significant difference in AP 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–P11: 287.70 ± 40.71 pA) compared with that in the younger age group (P0–P3: 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, whereas a subset of motoneurons expanded beyond this range. Some rheobase current measures of motoneurons from the older age group were up to fivefold 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 CV of rheobase current measures for each day in each age group and then compared the mean CV values. We found that there was a significant increase in the CV in the older age group (79.42%) compared with that of 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 1996, 2000). 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 AP firing rate regulation during repetitive firing (Fig. 3A). 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.

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**FIG. 4.** Input resistance (\(R_{in}\)) and AP frequency–current (\(f-I\)) relationships. **A1:** plot shows \(R_{in}\) measurements from each motoneuron at various postnatal ages. **A2:** comparisons of mean \(R_{in}\) and \(R_{in}\) CV between the younger (P0–P3) and older (P8–P11) age groups. **B1:** examples of repetitive AP firing in response of 2 levels of current injection. **B2:** comparisons of AP frequency–current relationships (\(f-I\) gains) and \(f-I\) gain CV values of younger (P0–P3) and older age groups (P8–P11); error bars ± SE.
In the present study, 20 consecutive AHP responses to a brief suprathreshold current injection (0.5 ms, 2–3 nA) were recorded, after which the AHP half-decay time was calculated as described earlier. 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 > 0.05, Fig. 3, A1 and A2). Although there was no significant difference in the mean AHP half-decay time, there was a significant increase in the CV 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 earlier text). Afterdepolarizations 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 (Rin) 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 Rin 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 Rin in the CV between the age groups: younger: 41.29%; older: 79.81%, P < 0.05), consistent with an increase in the CV of motoneuron excitability (Fig. 4A2).

Previous studies have noted a significant positive relationship between rheobase current and input conductance (Gin = I/Rin) 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 (r² = 0.28) between rheobase current and input conductance in the younger age group (P0–P3) and this relationship became somewhat stronger in the older age group (P8–P11, r² = 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–P3, P < 0.01; P8–P11, P < 0.01), but similar to Seebach and Mendell (1996), these slopes were not significantly different from each other (F = 0.003, P = 0.96).

No significant changes in f–I gain during neonatal development

Average AP firing rates were recorded in response to multiple suprathreshold, 2 s depolarizing current steps of varying amplitudes. Steady-state firing rates were calculated as the average AP firing rate of the last seven APs recorded for each current step (Fig. 4B1). The slope of the average firing rate in

![FIG. 5. Botulinum toxin type A (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).](http://jn.physiology.org/lookup/doi/10.1152/jn.00801.2008)
response to various current steps is reported as f–I gain. There was no statistically significant difference in the mean f–I gains (younger: 56.26 ± 3.03 Hz/nA; older: 56.09 ± 8.55 Hz/nA, P = 0.98, Fig. 4B2) or the associated CV values (younger: 32.03%; older: 33.59%, P = 0.92, Fig. 4B2) between the younger and older age groups.

All intrinsic property values for combined flexor and extensor motoneurons recorded from the younger (P0–P3) and older (P8–P11) age groups are reported in Table 1.

**BTX-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 (E) 12 and E14 in mice (Jansen and Fladby 1990). A number of studies have investigated various aspects of the process of NMJ formation and these studies have collectively illuminated mechanisms regarding trophic processes (Thesleff et al. 1990), apoptosis (Carr and Simpson Jr 1978), cell–cell signaling, synapse formation (Sanes and Lichtman 1999), activity-dependent synaptic plasticity, and homeostasis (Wang et al. 2005, 2006). 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 whether 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 hindlimb of postnatal mice (P0–P2, Fig. 5A1), before they began weight-bearing locomotion (Brown et al. 1982). The solution we injected into the extensor muscles included FG, which retrogradely labeled the motoneurons that innervate the extensor muscles (also see METHODS). 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 earlier. In a subset of experiments, EMGs were recorded from BTX-treated and control hindlimb muscles and we found that we could evoke no observable EMG signals in the BTX-treated hindlimbs (Fig. 5A2).

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

There were no significant differences in AP amplitude (control P0–P3: 79.67 ± 1.98 mV; control P8–P11: 72.62 ± 3.63 mV;
BTX P8–P11: 71.57 ± 2.23, ANOVA, P = 0.06, NS, Fig. 6A2) or AP threshold voltage (control P0–P3: −51.67 ± 1.94 mV; control P8–P11: −54.41 ± 2.16 mV; BTX P8–P11: −58.09 ± 2.01 mV, ANOVA, P = 0.08, NS, Fig. 6A5); moreover, there was no statistically significant difference in membrane capacitance between control P0–P3 (86.64 ± 7.271 pF), control P8–P11 (62.63 ± 4.243 pF), and BTX P8–P11 (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 earlier text). BTX-induced muscle paralysis led to a statistically significant increase in rheobase current compared with untreated, control extensor motoneurons. The mean rheobase current of extensor motoneurons after BTX treatment (P8–P11, 252.40 ± 46.31 pA) was significantly different from that of motoneurons from control animals at both P0–P3 (133.50 ± 14.54 pA) and P8–P11 (139.00 ± 20.54, ANOVA, P < 0.05, Fig. 7A), but there was no statistically significant difference in the CV of rheobase current (control P0–P3: 54.55%; control P8–P11: 59.18%; BTX P8–P11: 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–P3: 40.77 ± 4.94 ms; control P8–P11: 48.32 ± 4.96 ms; BTX P8–P11: 39.39 ± 4.30 ms, ANOVA, P = 0.43, NS, Fig. 7B) and CV values (control P0–P3: 39.33%; control P8–P11: 36.66%; BTX P8–P11: 47.11%, ANOVA, P = 0.78) that were statistically indistinguishable from those of motoneurons from either control age group (Fig. 7B).

Input resistance and f–I gain development during muscle paralysis

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 that of control extensor motoneurons in both age groups (control P0–P3: 158.30 ± 27.41 MΩ; control P8–P11: 156.20 ± 26.89; BTX P8–P11: 72.31 ± 9.46, ANOVA, P < 0.05, Fig. 8A), whereas there was no significant difference in input resistance CV (control P0–P3: 77.41%; control P8–P11: 69.52%; BTX P8–P11: 54.04%, ANOVA, P = 0.50, NS, Fig. 8A). Following BTX treatment, extensor motoneuron f–I gains from treated and controls had statistically indistinguishable f–I gains. There were no significant differences in the mean f–I gains (control P0–P3: 57.41 ± 7.16 Hz/nA; control P8–P11: 71.17 ± 9.41 Hz/nA; BTX P8–P11: 63.91 ± 13.31 Hz/nA, ANOVA, P = 0.54, NS, Fig. 8B) of the f–I gain CV (control P0–P3: 45.34%; control P8–P11: 39.49%; BTX P8–P11: 47.47%, ANOVA, P = 0.78, NS, Fig. 8B).

The intrinsic property values for control (P0–P3 and P8–P11) and BTX-treated (P8–P11) extensor motoneurons are reported in Table 2.

**DISCUSSION**

In this study we demonstrate that motoneuron-activated vesicle release and the resulting muscle activity lead 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 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: 1) that developmental diversification of motoneuron properties directly contributes to weight-bearing locomotion; 2) that weight-bearing locomotion (and the associated AP initiated muscle contractions, muscle activity) initiates mechanisms leading to motoneuron diversification; or 3) 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 hypoth-

**FIG. 8.** Input resistance and AP f–I relationships of control (P0–P3), control (P8–P11), and BTX-treated (P8–P11) extensor motoneurons; error bars ± SE. A: comparison of mean input resistance with statistically significant differences and input resistance CV values. B: comparison of mean AP frequency–current relationships (f–I gains) and CV values of f–I gains.
esis that precluding weight-bearing locomotion by paralyzing hindlimb extensor muscles with intramuscular BTX injections would affect the development and diversification of intrinsic motoneuron electrical properties. In our work, we observed that in extensor motoneurons inactivity led to an increase in the rheobase current. In adult rats tetrodotoxin (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 toward more negative values (although the resting membrane potential was also reduced). Thus our results fit with the general idea that activity promotes motoneuron excitability. In adults, BTX application generally mimics the effects of axotomy, which increase excitability by reducing the rheobase current and increasing the input resistance (Foehring and Munson 1983; Thompson et al. 1984). Furthermore, axotomy prolongs the time during which muscle fibers are polyneuronally innervated (Benoit and Changeux 1975, 1978; Caldwell and Ridge 1983; Thompson et al. 1984). That said, only some of their current injection amplitudes are sufficient for repetitive firing, which is probably greater than the minimum current sufficient to evoke a single AP. By dividing their data into two age groups—younger (P3–P5, n = 5) and older (P6–P11, 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, although the rheobase CV measures of the Fulton

**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–P12, n = 44 motoneurons). In the study by Fulton and Walton, a table is presented showing motoneurons and their measured 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 that reported by Fulton and Walton, there was no significant correlation between age and AP 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 AP half-width (our study: slope = −0.05 ms/day; Fulton and Walton: slope = −0.05 ms/day, r² = 0.12, P < 0.05). In contrast, the data reported by Fulton and Walton show a significant negative correlation between age and input resistance (r² = 0.45, P < 0.0001), whereas we found no statistically significant relationship (r² = 0.03, P = 0.06, NS). However, we did observe that the range of recorded values 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 the trend for a decrease was present, it was not statistically significant. Finally, the Fulton and Walton (1986) study provided some data (n = 10 motoneurons) on current injection and AP firing (i.e., rheobase current), although some of their current injection amplitudes are sufficient for repetitive firing, which is probably greater than the minimum current sufficient to evoke a single AP. By dividing their data into two age groups—younger (P3–P5, n = 5) and older (P6–P11, 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, although the rheobase CV measures of the Fulton

**TABLE 2. Motoneuron electrical properties recorded from untreated, control extensor motoneurons (P0–P3) control, (P8–P11), and botulinum toxin (BTX)–treated (P8–P11) extensor motoneurons**

<table>
<thead>
<tr>
<th>Property</th>
<th>Control P0–P3 (n = 16)</th>
<th>Control P8–P11 (n = 14)</th>
<th>BTX P8–P11 (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Action potential</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude, mV</td>
<td>79.67 ± 1.98</td>
<td>72.62 ± 3.63</td>
<td>71.57 ± 2.23</td>
</tr>
<tr>
<td>Half-width, ms</td>
<td>1.34 ± 0.18</td>
<td>1.53 ± 0.13</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>Time-to-peak, ms</td>
<td>0.87 ± 0.06</td>
<td>1.31 ± 0.15</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>Threshold, mV</td>
<td>−51.67 ± 1.94</td>
<td>−54.41 ± 2.16</td>
<td>−58.09 ± 2.01</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>
</tr>
<tr>
<td>Rheobase current CV, %</td>
<td>54.55 ± 59.18</td>
<td>72.98 ± 69.12</td>
<td>NS (0.57)</td>
</tr>
<tr>
<td>Afterhyperpolarization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Half-decay time, ms</td>
<td>40.77 ± 4.94</td>
<td>48.32 ± 4.96</td>
<td>39.39 ± 4.30</td>
</tr>
<tr>
<td>Half-decay time CV, %</td>
<td>39.33 ± 36.66</td>
<td>36.66 ± 47.11</td>
<td>47.11 ± 54.04</td>
</tr>
<tr>
<td>Input resistance (Rm), MΩ</td>
<td>158.30 ± 27.41</td>
<td>156.20 ± 26.89</td>
<td>72.31 ± 9.46</td>
</tr>
<tr>
<td>Rm CV, %</td>
<td>77.41 ± 69.52</td>
<td>54.04 ± 72.98</td>
<td>NS (0.50)</td>
</tr>
<tr>
<td>f-I gain, Hz/nA</td>
<td>57.41 ± 7.16</td>
<td>71.19 ± 9.41</td>
<td>63.91 ± 13.31</td>
</tr>
<tr>
<td>f-I gain CV, %</td>
<td>45.34 ± 39.49</td>
<td>47.49 ± 47.47</td>
<td>NS (0.78)</td>
</tr>
</tbody>
</table>

n = number of motoneurons. CV, coefficient of variation. * indicates significance; NS, not significant.
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 (1996) found a significant increase in rheobase current between younger [P1–P3: 1.0 ± 0.7 (SD) Na] and older [P7–P9: 2.9 ± 1.7 (SD) Na], with an accompanying increase in SD. 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 fibres is reduced compared with that of 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 fibres (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–P11). Specifically, the extensor group was more excitable than the mixed group, possibly due to a higher percentage of slow fibers in antigravity 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, in which motoneurons keep their rather long AHP over the first 2 wk (Fulton and Walton 1986). Therefore it would appear that, whereas 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 is 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 antigravity muscles because 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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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


