Increased Electrotonic Coupling in Spinal Motoneurons After Transient Botulinum Neurotoxin Paralysis in the Neonatal Rat

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Pastor, Angel M., George Z. Mentis, Rosa R. de la Cruz, Eugenia Díaz, and Roberto Navarrete. Increased electrotonic coupling in spinal motoneurons after transient botulinum neurotoxin paralysis in the neonatal rat. J Neurophysiol 89: 793–805, 2003; 10.1152/jn.00498.2002. The effect of early postnatal blockade of neuromuscular transmission using botulinum neurotoxin (BoNT) type A on motoneuron gap junctional coupling was studied by means of intracellular recordings and biocytin labeling using the in vitro hemisected spinal cord preparation of neonatal rats. The soma of tibialis anterior (TA) motoneurons were retrogradely labeled at birth (P0) by intramuscular injection of fluorescent tracers. Two days later, BoNT was injected unilaterally into the TA muscle. The toxin blocked neuromuscular transmission for the period studied (P4–P7) as shown by tension recordings of the TA muscle. Retrograde horseradish peroxidase tracing in animals reared to adulthood demonstrated no significant cell death or changes in the soma size of BoNT-treated TA motoneurons. Intracellular recordings were carried out in prelabeled control and BoNT-treated TA motoneurons from P4 to P7. Graded stimulation of the ventral root at subthreshold intensities elicited short-latency depolarizing (SLD) potentials that consisted of several discrete components reflecting electrotonic coupling between two or more motoneurons. BoNT treatment produced a significant increase (67%) in the maximum amplitude of the SLD and in the number of SLD components as compared with control (3.1 ± 1.7 vs. 1.4 ± 0.7; means ± SD). The morphological correlates of electrotonic coupling were investigated at the light microscope level by studying the transfer of biocytin to other motoneurons and the putative sites of gap junctional interaction. The dye-coupled neurons clustered around the injected cell with close somato-somatic, dendro-somatic and -dendritic appositions that might represent the sites of electrotonic coupling. The size of the motoneuron cluster was, on average, 2.2 times larger after BoNT treatment. Our findings demonstrate that a short-lasting functional disconnection of motoneurons from their target muscle delays motoneuron maturation by halting the elimination of gap junctional coupling that normally occurs during early postnatal development.

INTRODUCTION

In the developing nervous system, around the time when synaptic connections are established, neurons communicate with each other using both chemical and electrical transmission. Neuronal gap junctional communication has been demonstrated during embryonic and early postnatal development in various regions of the mammalian nervous system (Bennett 1977). One of the best-studied examples is the cerebral cortex of neonatal mammals where pyramidal neurons are extensively coupled via gap junctions (Peinado et al. 1993) forming neuronal clusters in columnar arrays (Yuste et al. 1995). The demonstration of coupling in several regions of the developing nervous system and, particularly, in the spinal cord suggests the possibility that gap junctions form functional compartments or neuronal domains that could play an important role in generating synchronous electrical and metabolic signals between the participating cells (Kiehn and Tresch 2002). In most regions of the CNS, coupling is transiently expressed during a particular period of development and declines sharply during maturation (Kandler and Katz 1995).

In neonatal rats, motoneurons supplying individual muscles are electrically coupled, and the incidence of this coupling decreases during the first two postnatal weeks (Chang et al. 1999; Walton and Navarrete 1991). The higher incidence of gap junctional coupling in immature motoneurons has been shown to result in synchronization of the motor output (Persson et al. 2001; Rekling and Feldman 1997) even in the absence of chemical synaptic transmission (Tresch and Kiehn 2000) and, therefore, has important functional consequences for motor control (Kiehn and Tresch 2002). Another factor that may contribute to the imprecise motor control during early development is the fact that individual muscle fibers are innervated by nerve terminals belonging to different motoneurons, resulting in overlap of the territories of different motor units (Brown et al. 1976; Redfern 1970). Thus synchronization of motoneuron activity and the presence of muscle polyneuronal innervation may limit the independent recruitment of motor units in developing animals (Navarrete and Vrbová 1993).

During the course of development, motoneuron maturation is dependent not only on activity-dependent orthograde influences from spinal interneurons and supraspinal descending pathways but also on retrograde influences arising from interactions between the motoneuron and its target muscle. Disconnection from the muscle in the early postnatal period as a result of peripheral nerve injury causes substantial loss of motoneurons, and those cells that survive display short- and long-term changes in dendritic morphology (Dekkers and Navarrete 2000) and, therefore, has important functional consequences for motor control (Kiehn and Tresch 2002). Another factor that may contribute to the imprecise motor control during early development is the fact that individual muscle fibers are innervated by nerve terminals belonging to different motoneurons, resulting in overlap of the territories of different motor units (Brown et al. 1976; Redfern 1970). Thus synchronization of motoneuron activity and the presence of muscle polyneuronal innervation may limit the independent recruitment of motor units in developing animals (Navarrete and Vrbová 1993).

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In the present study, we have examined the role of functional synaptic interaction with the target muscle in the postnatal maturation of identified tibialis anterior (TA) motoneurons. We first investigated the effect of functional blockade on motoneuron survival. We employed botulinum neurotoxin (BoNT) type A to block the quantal release of acetylcholine from motor nerve terminals (Kim et al. 1984; Molgo et al. 1989). We then examined the hypothesis that transient functional neuromuscular disconnection by BoNT might alter the developmental time course in the incidence and degree of electrotonic coupling in postnatal motoneurons. Finally, we have used morphological methods to investigate the extent of dye coupling and the putative sites of contact between the dye-coupled cells as well as aspects of motoneuron dendritic maturation.

Methods

Surgery

All surgical procedures were carried out in compliance with the UK Animal (Scientific Procedures) Act 1986. Motoneurons innervating the TA muscle were retrogradely labeled in vivo with a mixture of fluorescent tracers to allow their visual identification for intracellular recordings. Newborn rats (P0) were anesthetized with ether and the TA muscles were injected bilaterally with 1 µl of an aqueous suspension of 2.5% Fast Blue (FB) and 2.5% Diamidino Yellow–dihydrochloride (DY; EMS-Polyloy, Gross-Umstadt, Germany). Two days later (P2) the rats were re-anesthetized, and BoNT was injected only in the right TA muscle (unilateral injection), at a concentration of 0.1 ng/g of body wt (BoNT type A was kindly provided by Prof. O. J. Dolly, Imperial College London, UK).

In vitro hemisected spinal cord preparation and tension recordings

Animals were divided into three groups: control animals aged between P0 and P2, control animals (injected only with physiological saline) aged between P4 and P7, and BoNT-treated animals aged between P4 and P7. The first group of animals served as a reference to compare the time course of electrical coupling during development. The second group was used as the age-matched control group. For tension recordings, additional measurements were carried out in the contralateral side of the BoNT-treated animals.

On the day of the experiment, animals were anesthetized with ether and decapitated, and the vertebral column was transferred to a dissection chamber superfused with oxygenated Krebs solution at 10–15°C. The spinal cord was isolated through a ventral laminectomy and hemisected mid-sagittally. The preparation was mounted on the stage of an epifluorescence microscope (M2B, Microinstruments Oxford, UK) to visualize the prelabeled TA motoneuron pool and perfused at a rate of 3–5 ml/min with oxygenated Krebs solution maintained at room temperature (23–25°C). The Krebs solution consisted of (in mM) 113 NaCl, 4.5 KCl, 1 Mg2SO4, 2 CaCl2, 1 Na2HPO4, 25 NaHCO3, and 11 glucose.

In 27 preparations between P4 and P7, the sciatic nerve was dissected in continuity with the hindlimb and placed in the in vitro chamber equipped for tension recordings. The extensor retinaculum was cut at the ankle and the tendon of the TA muscle was secured to a strain gauge transducer to measure isometric force. In five of the preparations, tension was also recorded from the gastrocnemius and extensor digitorum longus muscles. A silver bipolar hook electrode was used for stimulation of the common peroneal nerve (indirect stimulation). In the case of gastrocnemius muscle recordings, the tibial nerve was stimulated. In addition, a bipolar electrode was placed forked around the muscle belly for direct stimulation. Care was taken to denervate all other calf muscles not used for tension recordings.

Intracellular recordings from identified motoneurons

Extracellular suction electrodes were used to either record or stimulate from the ventral roots, and bipolar hook electrodes were used to stimulate the dorsal roots. Ventral root (VR) recordings were performed using an AC-coupled preamplifier (Neurolog NL104, Digitimer, UK) with the bandwidth set at 0.1 Hz to 50 kHz. To assess the viability of the preparation, spinal reflexes were tested by stimulation of the dorsal root lumbar 4 (L4) while recording from the VR L4 (Fig. 1B) or VR L5. The retrogradely labeled TA motoneuron pool was visualized from the lateral aspect of the hemiscord under epifluorescence illumination. Addition of Lucifer yellow to the micropipette solution (Fig. 1, A and C) facilitated targeting of individual motoneurons for intracellular recordings (Fig. 1D). Images were captured using a CCD black/white video camera (WVBL-600 Panasonic) and stored in computer (RGB-Video, G2-Imaging, London, UK) for reference.

Intracellular recordings were carried out using glass micropipettes (Clark Electromedical) with a tip resistance of 15–25 MΩ. The microelectrodes were filled with a mixture of 0.03% of Lucifer yellow (dipotassium salt) and 2% biocytin (both from Sigma) made up in 1.5 M potassium acetate. At the end of the recording, the cell body and primary dendrites appeared labeled with Lucifer yellow, thus allowing subsequent mapping of the position of the impaled cell (Fig. 1, A and B).

![FIG. 1. Experimental design. A: micrograph showing Fast-Blue and Diamidino-Yellow-labeled pool of tibialis anterior (TA) motoneurons photographed during recordings. The somata of fluorescently labeled motoneurons were visualized using an ultra-violet filter (365–420 nm excitation wavelength). B: extracellular recording from the ventral root L4 after supramaximal stimulation (5 times threshold) of the dorsal root L4. C: visualization of an impaled motoneuron faintly labeled with Lucifer yellow. Scale bar represents 50 µm. D: intracellular recordings from a TA motoneuron. Stimulation at subthreshold intensity with respect to the antidromic spike demonstrated a short-latency depolarizing (SLD) potential (>). Recordings from a P6 treated preparation. Calibration bar is 0.25 mV and 50 ms for B and 25 mV and 10 ms for D.](http://jn.physiology.org/ Downloaded from http://jn.physiology.org/ by 10.220/32.246 on November 2, 2016)
motoneurons reported here compares well with the description of Peyronnard and Charron (1983). Furthermore, neither the somatic area (control: 1,466.6 ± 359.8 μm²; BoNT-treated: 1,513.6 ± 412.2 μm²) nor the average diameter (control: 44.2 ± 6.0 μm; BoNT-treated: 43.9 ± 6.2 μm) or the perimeter (control: 153.9 ± 20.4 μm; BoNT-treated: 152.7 ± 21.7 μm) differed significantly in BoNT-treated versus control motoneurons (P > 0.05, Student’s t-test). Finally, the distribution of motoneuron size, expressed as the mean of the large and small soma diameters, was similar for both control and treated motoneurons (P > 0.05, Kolmogorov-Smirnov 2-sample test). These results demonstrate that transient BoNT paralysis during early postnatal development does not result in neuronal death or long-lasting changes in somatic size of TA motoneurons.

### Time course of TA muscle paralysis

The isometric tension recorded from the TA muscle after electrical stimulation of the common peroneal nerve (indirect stimulation) was compared with that after stimulation of the muscle itself (direct stimulation) to determine the extent of paralysis (age range: P4–P7). A typical example of the paralysis caused by BoNT is illustrated in Fig. 3, A and B, for a P6 preparation after indirect stimulation in the control (arrows) or treated sides (baseline records). However, the tension measured after direct stimulation was similar in both the control and the treated muscle (Fig. 3, C and D). To account for muscle weight variability between animals, we normalized the results by calculating the percentage ratio of maximum tension produced by indirect stimulation (CP nerve) on direct stimulation (TA muscle). The histogram in Fig. 3E shows that, in the interval P4–P7 after BoNT injection at P2, the indirect/direct tension ratio was significantly decreased by 92.3% for the twitch tension and by 91.6% for the peak tetanic tension (at 40 Hz; P < 0.001, 1-way ANOVA, Tukey test). By comparison, in the contralateral-control TA muscle from BoNT-treated animals, the indirect/direct tension ratio was virtually the same that obtained in control untreated animals (for both twitch and tetanic tension), indicating that there were no systemic effects of the toxin in the contralateral-control side of BoNT-treated animals (Fig. 3E). Addition of 10 μM curare to the bath solution in two control P6 preparations (data not shown) resulted in a 100% tension reduction after indirect stimulation as expected and a 19.3% reduction after direct stimulation. This latter reduction represents the neuromuscular contribution to tension during direct stimulation, presumably blocked in treated preparations (Fig. 3E).

### Results

#### Long-term survival of TA motoneurons

To assess the long-term effect of transient neonatal paralysis on motoneuron survival and morphometry, six P2-treated animals were reared into adulthood and HRP-labeled TA motoneurons on both sides of the spinal cord were analyzed. In transverse sections, the TA motoneuron pool appeared as a small cluster in the dorsolateral part of the ventral horn, adjacent to the white matter of the lateral funiculus in both control and BoNT-treated sides (Fig. 2, A and B). Horizontal sections revealed that the labeled TA motoneuron pool spanned the whole L₄ and rostral L₅ lumbar spinal cord segments (Fig. 2, C and D). The number of motoneurons in the operated side of the spinal cord (148.5 ± 22.3; mean ± SD) was not significantly different (P > 0.5, paired Student’s t-test) to that on the contralateral control side (140.7 ± 30.6). The number of TA
Electrotonic coupling in control and BoNT-treated motoneurons

To determine the effect of BoNT paralysis on the electrical coupling of individual TA motoneurons, intracellular recordings were obtained from fluorescently prelabeled (FB/DY) TA motoneurons in control (untreated) and BoNT-treated animals. Figure 4A shows the effect of graded stimulation of the VR L4 in a labeled TA motoneuron from a BoNT-treated P6 animal (see also Fig. 1D). Suprathreshold stimulation elicited an antidromic action potential demonstrating invasion of the somatodendritic compartment. Decreasing the stimulus intensity to a level just below that required for antidromic activation demonstrated the presence of a small depolarizing potential (Fig. 4A, 2). The subthreshold nature of the recorded depolarizing potentials and its short-latency with respect to the onset of the antidromic spike indicated that they originate from electrotonic interactions between neighboring motoneurons belonging to the same or synergistic motoneuron pools (Walton and Navarrete 1991). In the majority of cases, the short-latency depolarizing (SLD) had a longer latency compared with the antidromic spike (Figs. 4 and 5), but in a few cases, the opposite was evident (not illustrated).

Properties of electrotonic potentials

Several tests were employed to demonstrate that the SLDs were due to the activation of spikes generated in the myelinated axon (the M spike) of the recorded motoneuron, we performed collision tests between orthodromically and antidromically conducted action potentials. As shown in Fig. 4B, a conditioned orthodromic action potential elicited by passing a pulse of inward current through the microelectrode prevented the appearance of the antidromic action potential using suprathreshold stimulus intensity, demonstrating the presence of an SLD (Fig. 4B; ●). The latency of SLDs in BoNT-treated TA motoneurons (P4–P7) did not differ from their age-matched controls (BoNT: 4.2 ± 2.1 ms; control: 4.1 ± 1.8 ms). However, the maximum SLD amplitude was significantly greater in BoNT-treated cells (BoNT: 2.0 ± 0.8 mV; control: 1.2 ± 0.6 mV, P < 0.05, Student’s t-test). Furthermore, the time-to-peak (tp) and time-to-half-decay (thd) were significantly reduced in BoNT-treated cells (tp: 2.5 ± 0.7 ms; thd: 9.5 ± 6.4 ms) compared with their age-matched controls (tp: 3.5 ± 1.7 ms; thd: 13.4 ± 5.4 ms; P < 0.05, Student’s t-test). The input resistance of impaled motoneurons was assessed after injection of depolarizing and hyperpolarizing current pulses (100 ms duration) at the resting membrane potential and calculated from the slope of the current/voltage plot within the linear range. BoNT-treated motoneurons revealed similar values of input resistance compared with their age-matched controls (control: 7.3 ± 4.4 MΩ, n = 9; BoNT-treated: 6.9 ± 2.9 MΩ; n = 12). Thus the properties (tp and thd) of SLDs in treated
found a reduction of coupling in control TA motoneurons during normal development (Table 1; P0–P2 vs. P4–P7 control cells). However, blockade of neuromuscular transmission by the toxin halted this normally occurring reduction in electrotonic coupling (Table 1; control vs. treated cells at P4–P7). The number of discrete SLD components was relatively high in the neonatal TA motoneurons (100% incidence in electrotonic coupling). Although the incidence was similar, the number of discrete SLD components was significantly greater ($P < 0.05$, 1-way ANOVA on Ranks, Dunn’s test) in BoNT-treated motoneurons ($3.1 \pm 1.7$) compared with their age-matched control motoneurons ($1.4 \pm 0.7$). However, there was no significant difference between the P4–P7 BoNT-treated group and the P0–P2 control group of cells (Table 1). As stated in the preceding text, the maximum SLD amplitude in treated motoneurons was significantly larger than in controls. Nonetheless, the amplitude of discrete SLD components was similar between the control and BoNT-treated motoneurons. Thus the mean value of the first component was $0.95 \pm 0.44$ mV in control and $1.06 \pm 0.55$ mV after BoNT. When measurements were carried out for all resolvable components, the mean amplitude of discrete SLD components was $0.83 \pm 0.48$ mV in control and $0.59 \pm 0.51$ mV in BoNT-treated motoneurons. In both cases, differences were not significant ($P > 0.5$ and $P > 0.1$, respectively; Student’s t-test). This finding suggests that the difference obtained in the maximum amplitude of SLDs was due to a higher number of SLD components in BoNT-treated motoneurons.

The SLDs were resistant to the application of transmembrane currents. Alterations in the membrane potential by cur-

motoneurons suggest a closer location to the recording site (the soma) of the interaction with coupled motoneurons.

Graded stimulation of the ventral root within the subthreshold level revealed the presence of several discrete components of the SLD that increased gradually in amplitude until the firing threshold for antidromic activation of the impaled motoneuron was reached (Fig. 5A). Blockade of firing of the impaled cell by the preceding-described tests demonstrated in some instances further components.

To evaluate the effect of BoNT paralysis on the developmental loss of electrotonic coupling in TA motoneurons, we performed intracellular recordings from younger preparations (P0–P2). The results are shown in Table 1. As previously reported (Chang et al. 1999; Walton and Navarrete 1991), we

FIG. 3. Extent of neuromuscular transmission block after BoNT treatment. A and B: isometric tension recordings from the TA muscle after indirect stimulation via the common peroneal nerve using a single shock (A) or a 1-s train at 40 Hz (B) in a control (↓) and a BoNT-treated side (baseline traces) from a P6 preparation. C and D: same as A and B but after direct stimulation of the muscle in the same preparation. ↓, the control traces (contralateral-control TA muscle). •, onset of stimulation. E: normalized tension measurements (mean ± SD) expressed as the indirect (CP nerve) on direct (TA muscle) tension ratio recorded from the contralateral control side in 4 animals at P4–P7 and from the BoNT-treated side in 19 animals and in 4 control untreated (normal) animals. Note that the ratio of indirect to direct tension for twitch and tetanic stimulation was significantly reduced in BoNT-treated muscles ($P < 0.001$, 1-way ANOVA, Tukey test). F: time course of muscle paralysis induced by BoNT expressed as the indirect/direct ratio of the peak twitch and tetanic tension (mean ± SD), showing that an almost complete neuromuscular transmission block is maintained during the 1st 5 days after BoNT injection at P2. Five animals per group except at P5 (4 animals).

FIG. 4. Electrotonic coupling in TA motoneurons. A: antidromic spike evoked in a P6 BoNT-treated TA motoneuron by stimulation of the VR L4 at a shorter latency than its short-latency depolarizing (SLD, ↓). Inset: a higher magnification of the SLD at the base of the spike. a, the resting potential. •, the onset of the stimulus artifact. B: insensitivity of SLD to collision test. Three traces are superimposed, corresponding to the antidromic action potential evoked after stimulating the VR L4 (a), the underlying SLD evoked by subthreshold VR stimulation (↑), and the collision of the antidromic spike with a preceding orthodromic spike elicited by intracellular current injection (●). Recordings were carried out in a P5 BoNT-treated preparation.

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ventral root L 4 at increasing strength demonstrated the gradation of the SLD.

6-cyano-7-nitroquinoxaline (CNQX, 5 μM) to block glutamatergic transmission and picrotoxin (100 μM) to block GABA and glycinergic transmission. After the addition to the bath of APV (200 μM) and CNQX (5 μM) to block glutamatergic transmission and picrotoxin (100 μM) and strychnine (10 μM) to block GABA and glycinergic transmission, the amplitude of the SLD remained constant (Fig. 5D). A similar result was obtained when bathing the preparation in 0 mM Ca²⁺.

Dye coupling

Because electrical coupling mediated by gap junctions is often accompanied by passage of small molecular weight tracers, as has been shown in other parts of the nervous system (Peinado et al. 1993), we studied the incidence of dye coupling in the electrophysiologically characterized cells by electrophoretic injection of biocytin. As expected, the histochemical detection of biocytin revealed the tracer not only in the impaled cell (Fig. 6B) but also in a discrete group of cells that formed a cluster around the injected master cell and in close proximity.

TABLE 1. Electrotonic and dye coupling in control and BoNT-treated motoneurons

<table>
<thead>
<tr>
<th>Electrotonic Coupling</th>
<th>Dye Coupling</th>
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<tr>
<td></td>
<td>Control (P0–P2)</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
</tr>
<tr>
<td>Incidence, %</td>
<td>100</td>
</tr>
<tr>
<td>Components</td>
<td>5.3 ± 2.1</td>
</tr>
<tr>
<td>Range</td>
<td>1–3</td>
</tr>
</tbody>
</table>

Incidence represents the percentage of motoneurons that exhibited electrotonic or dye coupling. The number of discrete short-latency depolarizing (SLD) components or number of coupled cells is shown for those motoneurons in which coupling was present (mean ± SD). The corresponding ranges are shown in parenthesis. Statistical test was one-way ANOVA on Ranks, Dunn’s test: * P < 0.05 vs. control (P0–P2), † P < 0.05 vs. control (P4–P7). BoNT, botulinum neurotoxin.
to its dendrites (Fig. 6, A and C). The identification of the impaled motoneuron was cross-checked against the information acquired from photographs during recording, the relative distance from the edge of the spinal cord, the intensity of biocytin labeling and the extent of its dendritic tree. On occasion, the biocytin visualization also delineated the axon of coupled cells directed toward the VR. The impaled cell had in most cases a complete delineation of the entire dendritic tree extending radially by more than 600 μm. (Fig. 6C). The extent of biocytin filling allowed also the visualization of the axon collaterals as one to four fine axonal sprouts directed dorsally toward the motor nucleus (Fig. 6C).

The physiological detection of electrotonic coupling seemed to be a more sensitive method than the dye-coupling method. Occasionally, although the injected cell was entirely visualized, no coupled cells were detected, even though the electrotonic coupling was demonstrated. Despite these facts, on average, both the anatomical and the electrophysiological methods detected about equal number of coupled cells in the control and treated groups. For example, in control motoneurons, an average of 1.4 ± 0.7 components were found in the SLD and a similar number of cells (1.5 ± 0.5) were dye-coupled (Table 1). Although the incidence of dye coupling was similar, the cluster size was significantly larger in BoNT-treated motoneurons as compared with their age-matched controls (Table 1, \( P < 0.05 \), 1-way ANOVA on Ranks; Dunn’s test).

**Location of the anatomical coupling**

We employed the dye-coupling method in this study because it would provide important information about the identity and distribution of clusters of coupled cells as well as the putative sites of gap junctional communication, although a definitive assessment of the actual sites of dye coupling would require the use of electron microscopic analysis. The location and distance of the dye-coupled cells in relation to the impaled motoneuron for all the cells included in this study was measured with the aid of camera lucida drawings as illustrated in Fig. 7A. Coupled motoneurons were located around the impaled cell, usually...
within two tissue sections (200 μm), in a similar manner in both control and treated preparations. The mean distance of coupled cells to the impaled cell was 146 ± 97 μm (n = 22) in control and 118 ± 72 μm (n = 45) in treated preparations. Two-dimensional plots (all clusters having the impaled cell located in the origin of coordinates) revealed that the dye-coupled cells were aligned along the rostrocaudal axis of the motoneuronal pool (Fig. 7A). Approximately 76% of the control dye-coupled motoneurons and 60% of the BoNT-treated dye-coupled motoneurons were located in angle sectors directed rostrally and caudally to the impaled cell (Fig. 7B). This result is consistent with the fusiform shape of the TA motoneuron pool (Fig. 1A).

At the light microscope, it was possible to find one or more sites of close apposition (putative sites of interaction) between the impaled and the coupled motoneuron. Only 3.5% of the appositions in control and 1.5% in treated preparations occurred between the somata of the impaled and the coupled cell (somato-somatic; Table 2), suggesting that motoneurons were not accidentally labeled along the electrode track during recordings. Moreover, a significantly larger than control (P < 0.001, χ² test) and approximately half (48.5%) of all appositions in treated preparations were between distal dendrites (i.e., third-order dendrites and higher) of the injected cell and the soma of the coupled motoneuron (Table 2). By contrast, control preparations exhibited a significantly larger number of distal dendrite to second-order dendrite interactions (P < 0.01, χ² test). Because many of the dendro-dendritic categories had zero representatives in the treated group (Table 2), we grouped these putative sites of interaction as dendro-dendritic or somatic. Thus dendro-dendritic interactions were 82.4% in control and 26.5% in BoNT-treated, whereas dendro-somatic were 8.8% for control and 70.5% in BoNT-treated. The control group presented a significantly larger number of dendro-dendritic interactions, whereas the BoNT-treated group had a larger number of dendro-somatic interactions (P < 0.001, Fisher exact test).

Frequently, a dendrite originating from the impaled cell was found in close apposition to the soma of a dye-coupled motoneuron (Fig. 8A) and climbed over a proximal dendrite trunk (Fig. 8, B and C). In many cases, the putative site of coupling consisted of a number of thin and elaborated appendages emerging from the dendrite of the impaled cell toward the coupled motoneuron. As seen in Fig. 8D, a heavily stained distal dendrite from the impaled cell curved around a primary dendritic trunk of a coupled motoneuron and emitted several, spine-like, appendages directed at several points toward the coupled motoneuron. In other cases, those appendages appeared in a thickened swelling of a dendrite with many thin processes oriented toward the coupled cell (Fig. 8A). Furthermore, another observation of putative coupling consisted of a bundle of fine, long, dendrite-like appendages that ramified from primary or secondary dendrites and that appeared to embrace a coupled cell (Fig. 8B).

**DISCUSSION**

The present experiments demonstrate that transient functional disconnection of the motoneuron from its target muscle during early development using BoNT results in an arrest or delay in the elimination of electrotonic coupling that normally occurs during early postnatal development (Chang et al. 1999; Walton and Navarrete 1991). BoNT treatment did not result in motoneuron death or in significant changes in somatic size. In this study, we have shown that dye transfer accompanies electrotonic coupling in neo-

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**TABLE 2. Sites of interaction between coupled spinal motoneurons**

<table>
<thead>
<tr>
<th>Coupled Cell Compartment</th>
<th>Injected Cell Compartment</th>
<th>Soma</th>
<th>1st-Order Dendrite</th>
<th>2nd-Order Dendrite</th>
<th>Distal Dendrite</th>
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<tr>
<td></td>
<td>Con</td>
<td>BoNT</td>
<td>Con</td>
<td>BoNT</td>
<td>Con</td>
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<tr>
<td>Soma</td>
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<td>8.8</td>
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</tr>
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<td>2nd-order dendrite</td>
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<td>0</td>
<td>0</td>
<td>10.5</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>10.5</td>
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Numbers are the percent of the total scored interactions: n = 57 interactions in 10 clusters of control (Con) and n = 68 interactions in 12 clusters of treated (BoNT) motoneurons. * Significantly (P < 0.01) larger proportion of distal dendrite to second-order interactions in control preparations; ** Significantly (P < 0.001) larger proportion of distal dendrite-to-soma interactions in BoNT-treated preparations (χ² test).
natal ankle flexor motoneurons between the coupled neurons. Graded stimulation of the ventral root resulted in multiple components of the SLD potential corresponding to recruitment of axons from one or more motoneurons coupled to the impaled cell (Walton and Navarrete 1991). The mean number of dye-coupled neurons was similar to the number of SLD components suggesting that the electrotonically coupled cells were linked by gap junctional connections.

**Properties of the electrotonic coupling**

The existence of gap junctional coupling between clusters of TA motoneurons at neonatal stages is strongly supported by the findings of the present study. At the physiological level, the presence of graded, subthreshold, SLDs represent the strongest evidence of electrotonic coupling between a cluster of motoneurons, given that a direct demonstration, that is, the simultaneous recording of two coupled cells was not employed here (Logan et al. 1996; Rekling and Feldman 1997). The demonstration of biocytin-labeled cells other than the injected motoneuron and the presence of sites of close apposition between the impaled motoneuron and the coupled cells provide further evidence (although ultrastructural evidence would be unequivocal evidence) for the presence of gap junctional coupling between motoneurons of the neonatal rat spinal cord. Moreover, the similarity of cluster size obtained from physiological and morphological data suggests that an accurate prediction of the cluster size of coupled motoneurons can be inferred from the number of resolvable components in the SLD demonstrated by means of graded stimulation.

**FIG. 8.** Putative sites of interaction between coupled cells. **A**: dendro-somatic interaction between a dendrite of the injected motoneuron directed in the rostrocaudal direction and 2 coupled motoneurons in a P6 preparation. Note the thickenings in the dendrite with processes over the coupled motoneurons. **B**: several cells contacted by the dendrites of an impaled motoneuron whose soma is in a different section in a P4 preparation. **C**: a distal dendrite of an impaled motoneuron at P7 runs over the soma and a primary dendrite of a coupled motoneuron. **D**: interaction between thin spine-like appendages of a distal dendrite of the impaled motoneuron with a primary dendrite of a coupled motoneuron at P5. All preparations were treated with BoNT. Calibration bars are 15 μm in A, 25 μm in B, 20 μm in C, and 10 μm in D.
Presumptive sites of gap junctional coupling

Several arguments indicate that dye-coupled cells were motoneurons. First, their size was similar to that of the impaled motoneuron and they were also located within the boundaries of the TA pool as indicated by the FB/DY labeling of the cells (data not shown). Furthermore, in some instances, dye-coupled cells also showed a faintly delineated axon extending into the ventral root. These results suggest that dye coupling is spatially organized and reinforce the conclusion that electrotonic interactions occur predominantly in functionally related cells (Walton and Navarrete 1991). Similarly, electrophysiological tests have shown sympathetic preganglionic neurons to be electrotonically coupled to other sympathetic neurons (Logan et al. 1996). Furthermore, soleus motoneurons in young adult rats have been shown to possess gap junctions between their dendrites indicative of electrotonic coupling (van der Want et al. 1998).

During early postnatal development, the cell bodies of retrogradely labeled motoneurons are tightly clustered with the somata of adjacent motoneurons found in direct apposition with no intervening neuropil discernible at the light microscope (Kerai et al. 1995). In addition, the dendrites of motoneurons belonging to the same pool form extensive bundles extending rostrocaudally and laterally into the lateral luniculus (Scheibel and Scheibel 1971; Westerga and Gramsbergen 1992). These may represent potential sites of contact between coupled cells as a higher incidence of gap junctions have been found in dendritic bundles of adult motoneurons (Kerns and Peters 1974; Matsumoto et al. 1988; van der Want et al. 1998). In the present study, we found several sites of close contact between the coupled cells that may represent the sites of cellular interactions, i.e., somato-somatic, dendro-somatic and -dendritic. An anatomical study of gap junctions between bulbocavernousus motoneurons in the adult rat revealed the presence of gap junctions in all these areas with the majority (45%) being found in somato-dendritic sites (Matsumoto et al. 1988). In addition, recent work by Chang and colleagues (1999) has demonstrated the presence of connexins 36, 37, 40, 43, and 45 in neonatal rat spinal cord motoneurons. It is interesting that the putative site of coupling often consisted of growth-cone-like appendages emerging from the dendrite of the impaled cell that interacted with parallel processes of the coupled motoneuron. This observation suggests the possibility that filopodial growth-associated processes of bundling dendrites may contact each other during their parallel course. At the ultrastructural level, Motorina (1989) reported very few gap junctions in neonatal rats and the majority of these were somato- and dendro-dendritic in nature. Further studies should explore in more detail the ultrastructural features associated with gap junctional coupling in neonatal animals.

In this study, the highest incidence of putative sites of contact were dendro-somatic in treated motoneurons compared with their control counterparts where only a small percentage was scored as dendro-somatic. According to these observations it is tempting to speculate that the normal elimination of gap junctions would take place in a somatofugal direction which parallels that of dendritic maturation (Dekkers et al. 1994). Short- and long-lasting effects of BoNT blockade of neuromuscular transmission

It has been previously reported that neonates are more resistant to neuromuscular blockade induced by BoNT injection as compared with adult animals, but the reasons for this phenomenon are not clear. After BoNT type A treatment in 1- to 3-wk-old rats, complete neuromuscular blockade lasts for approximately 3–5 days (Bambrick and Gordon 1989; Brown et al. 1981), and the present experiments confirm these previous findings. We have further shown that the transient neuromuscular transmission block is circumscribed to the injected muscle as the contralateral TA and other ipsilateral muscles (extensor digitorum longus, gastrocnemius) were not significantly affected after a single neonatal intramuscular injection of BoNT. The effective dose was similar to that reported in adult cat abducens motoneurons (Moreno-López et al. 1997; Pastor et al. 1997). In this study, the dose used was the maximum tolerated, whereas smaller dosages resulted in partial neuromuscular blockade (data not reported). Although by the use of repeated injections it was possible to prolong the period of neuromuscular block (Brown et al. 1981; unpublished observations), we avoided this to prevent damage to the small neonatal TA muscle and/or its innervation.

The neonatal treatment with BoNT did not alter the survival of motoneurons up to the adult stage. These results are also consistent with those of a previous study in which neuromuscular transmission was blocked at the postsynaptic level using α-bungarotoxin in neonates. In that case, neither the survival nor the rate of increase in the somatic size of soleus motoneurons was affected during the first three postnatal weeks (Kerai et al. 1995). It would, therefore, appear that functional disconnection from the target has different effects on cell survival from physical disconnection induced by axotomy, where significant cell loss is observed (Lorrie et al. 1987; Schmalbruch 1984). This discrepancy suggests that cell death after axotomy might be related not to the loss of functional target connection but rather to the lack of some retrograde factor that is not impeded in the BoNT-treated motoneurons.

Effects of BoNT on coupling

By using BoNT to block the neuromuscular synapse, we demonstrated that the extent of motoneuron electrotonic coupling and its dendritic maturation in dependent on the functional interaction with the target muscle. The size of the cluster of coupled motoneurons was larger in animals treated at P2 with BoNT than in control motoneurons. In agreement with this, a larger number of resolvable components of the SLD and a larger maximum SLD amplitude was found in treated motoneurons.

Little is known about the factors that control the extent of electrical coupling during early development. Previous experiments using postsynaptic blockade of neuromuscular transmission have demonstrated that orthograde synaptic activity is involved in regulating electrical coupling between muscle cells (Armstrong et al. 1983). The neurotransmitters glutamate (Pereda and Faber 1996) and serotonin (Rorig and Sutor 1996) have also been shown to acutely modulate the permeability of gap junctions via second-messenger systems that include cal-
cium and calmodulin kinase II (Bruzzone and Ressot 1997). It is possible that, in the longer-term synaptic activity could regulate the degree of coupling by means of neurotransmitter-related second-messenger signals leading to alteration in the levels of gap junctional proteins. The fact that motoneuron coupling decreases at the time when spinal circuits involved in locomotor activity become increasingly driven as a result of functional maturation of descending pathways (Clarac et al. 1998; Navarrete and Vrbová 1984; Navarrete et al. 2002; Westerga and Gramsbergen 1992) suggests that an increase in afferent synaptic activity may be involved in the development of downregulation of gap junctional coupling. Indeed, recent results show that transient blockade of the NMDA subtype of glutamate receptors in early postnatal development delay the postnatal elimination of gap junctional coupling between spinal motoneurons (Mentis et al. 2002).

The present results showing that the normal maturation of electrotonic coupling (i.e., their progressive elimination with age) is either arrested or significantly delayed by blockade of neuromuscular activity together with those of a parallel study in axotomized neonatal TA motoneurons showing an increase in the cluster size of electrotonically coupled cells (Mentis et al. 1996; unpublished data) therefore provide strong new evidence for an activity-dependent retrograde regulation of motoneuron coupling in the somatodendritic domain.

Relationship to motoneuron growth

During the first 2 wk of postnatal development, there are important changes in the growth status of the motoneuron as indicated by a substantial remodeling of both the axonal and dendritic fields. The early postnatal period is characterized by a pattern of gene expression conducive to growth, as indicated by high levels of expression of the growth-associated proteins GAP 43 and CAP 23 (Caroni 1997; Chong et al. 1992; Laux et al. 2000). At birth, the axonal peripheral field is maximally expanded, the motor unit territory being up to times larger than in the adult, and individual muscle fibers are polynuronal innervated (Brown et al. 1976). At this time, the somatodendritic domain contains large numbers of growth-associated processes (e.g., spines, filopodial, and lamellipodial growth cones). During the second postnatal week, these growth-associated processes are eliminated in a somatofugal manner (Dekkers et al. 1994). These events occur concurrently with the elimination of muscle polynuclear innervation (Brown et al. 1976), and the reduction of gap junctional coupling (Chang et al. 2000; Personius and Balice-Gordon 2001; Walton and Navarrete 1991) and are associated with a developmental downregulation of growth-associated proteins (see Caroni 1997).

It is known that BoNT paralysis in neonates results in restoration of polynuclear innervation at the neuromuscular junction, presumably due to reactivation of axonal branches destined to be eliminated (Brown et al. 1981). Furthermore, both axotomy and blockade of neuromuscular transmission prevents the developmental downregulation of genes associated with neuronal growth (Caroni and Becker 1992; see Caroni 1997). This suggests, that events associated with functional interaction between the motoneuron and its target muscle regulate in a retrograde fashion the maturation of motoneuron dendrites and their axonal terminal fields, as also previously shown in the sympathetic nervous system (Purves et al. 1988; Voyvodic 1987).

It is possible that the reactivation of axonal (Brown et al. 1982) and dendritic growth induced by blockade of neuromuscular transmission may also increase the likelihood of maintaining sites of dendritic gap junctional contacts. Very little information is available about the distribution of gap junctional contacts in immature motoneurons, but it is interesting that in the adult spinal cord, gap junctions are often present in conjunction with chemical synapses (mixed synapses) as demonstrated by Rash et al. (2000). During early synaptogenesis in the spinal cord, dendritic growth cones are preferential sites of chemical synaptic inputs (see Vaughn 1989), and thus the intriguing possibility of a preferential distribution of gap junctional proteins at sites of dendritic growth (suggested by the present results) should be investigated at the ultrastructural level. The cytoskeletal protein actin is a major structural component of filopodial growth cones, and this protein is also associated with cell-cell signaling junctions such as focal contacts and adherent junctions. Actin is also associated with gap junctional complexes in mixed synapses of goldfish Mauthner cells (Moshkov et al. 1998), and there is also evidence that disruption of microfilaments using drugs that depolymerize actin inhibit the clustering of connexins 43 within gap junctional complexes (Wang and Rose 1995). Due to its unique mechanochemical properties, dynamic changes in the actin cytoskeleton may play an important role in the rearrangements of cell-cell communication during development and in adult plasticity (Harris 1999). In conclusion, the similarity in the findings after both experimental manipulations suggests that the electrical activity at the neuromuscular synapse, or at the muscle itself, appears to be a critical factor controlling the development of both electrical coupling and dendritic growth-associated processes.

Role of gap junctional coupling in neuromuscular development

It is well established that the motoneuron firing pattern plays an important role in regulating the muscle contractile properties and its pattern of innervation (see Navarrete and Vrbová 1993). The presence of electrotonic coupling has been shown to result in synchronization of motoneuron firing in the spinal cord (Rekling and Feldman 1997; Tresch and Kiehn 2000), and it might therefore be expected that a delay in the time course of its elimination may have consequences for neuromuscular development. Blockade of neuromuscular activity leads to a reduction in the rate of elimination of polynuclear innervation (Brown et al. 1981; Srinhari and Vrbová, 1978; Thompson et al. 1979), while peripheral nerve electrical stimulation accelerates the loss of polynuclear innervation (O’Brien et al. 1978). Recent experiments on developing and adult reinnervated muscle (Busetto et al. 2000; Personius et al. 2001) strongly suggest that the temporal patterns of activity of motoneurons converging to a given set of target muscle fibers play a key role in controlling the process of competitive synapse elimination at the neuromuscular junction by means of Hebbian mechanisms previously shown to operate in the developing visual system (for review, see Zhang and Poo 2001). Suppression of the normal pattern of motoneuron activity by focal tetrodotoxin
blockade of nerve conduction was shown to prevent the elimination of polyneuronal innervation in adult reinnervated muscles, and a similar effect was observed when the axons were activated synchronously distal to the block by electrical stimulation (Busetto et al. 2000). In addition, it has recently been shown that the degree of synchronization of motoneuron activity becomes reduced in parallel with the elimination of polyneuronal innervation and could drive the activity-dependent process of elimination of polyneuronal innervation (Peronis et al. 2001).

Finally, the present results demonstrating that synaptic activity at the neuromuscular junction regulates in a retrograde manner electrotonic interactions between functionally related motoneurons at the level of the somatodendritic domain may be important for our understanding of the mechanisms controlling motoneuron functional maturation. Previous studies in adult animals have shown that alterations in the level of activity at the neuromuscular junction influences motoneuron membrane excitability and dendritic architecture (Czech et al. 1978; Sumner and Watson 1971). Thus it may be suggested that alteration in the growth status of the motoneuron nerve terminals in the peripheral may regulate the morphological and functional differentiation of the cell.

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