Electrical stimulation of transplanted motoneurons improves motor unit formation

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Liu Y, Grumbles RM, Thomas CK. Electrical stimulation of transplanted motoneurons improves motor unit formation. J Neurophysiol 112: 660–670, 2014. First published May 21, 2014; doi:10.1152/jn.00806.2013.—Motoneurons die following spinal cord trauma and with neurological disease. Intact axons reinnervate nearby muscle fibers to compensate for the death of motoneurons, but when an entire motoneuron pool dies, there is complete denervation. To reduce denervation atrophy, we have reinnervated muscles in Fisher rats from local transplants of embryonic motoneurons in peripheral nerve. Since growth of axons from embryonic neurons is activity dependent, our aim was to test whether brief electrical stimulation of the neurons immediately after transplantation altered motor unit numbers and muscle properties 10 wk later. All surgical procedures and recordings were done in anesthetized animals. The muscle consequences of motoneuron death were mimicked by unilateral sciatic nerve section. One week later, 200,000 embryonic day 14 and 15 ventral spinal cord cells, purified for motoneurons, were injected into the tibial nerve 10–15 mm from the gastrocnemius muscles as the only neuron source for muscle reinnervation. The cells were stimulated immediately after transplantation for up to 1 h using protocols designed to examine differential effects due to pulse number, stimulation frequency, pattern, and duration. Electrical stimulation that included short rests and lasted for 1 h resulted in higher motor unit counts. Muscles with higher motor unit counts had more reinnervated fibers and were stronger. Denervated muscles had to be stimulated directly to evoke contractions. These results show that brief electrical stimulation of embryonic neurons, in vivo, has long-term effects on motor unit formation and muscle force. This muscle reinnervation provides the opportunity to use patterned electrical stimulation to produce functional movements.

muscle reinnervation; axon regeneration; muscle force

MOTONEURON DEATH OCCURS with spinal cord injury, with various neurological diseases such as poliomyelitis and amyotrophic lateral sclerosis, and with natural aging (Bradley et al. 1983; Doherty et al. 1993; Sharrard 1955; Thomas and Zijdewind 2006). Although the mechanisms of motoneuron death may differ in each of these conditions, they all result in denervation of muscle. Muscle fibers atrophy and weaken as a result of denervation. Denervated muscles also become less excitable, fatigable, and contract and relax more slowly (Ashley et al. 2007; Finol et al. 1981; Gunderson 1985; Gutmann 1948; Hennig and Lomo 1987; Kotsias and Muchink 1987). Sprouting of intact intramuscular axons is an important compensatory mechanism to restore muscle innervation and function after partial denervation of muscle. If the initial motoneuron death is extensive, or progresses, the limits of axon sprouting may be reached, leaving some muscle fibers denervated chronically (Brown et al. 1981; Gordon et al. 1993; Thompson and Jansen 1977). One strategy to arrest denervation atrophy is to electrically stimulate muscle. When stimulation is initiated immediately after denervation and delivered every day or on weekdays in small rat, rabbit, and cat muscles, muscle properties recover to 37–85% of uninjured values (Dow et al. 2005; Hennig and Lomo 1987; Westgaard and Lømo 1988). However, when denervated human muscle is stimulated 6–12 mo after spinal cord injury and continued for 1–8 yr, the larger muscle fibers are restricted to the vicinity of the stimulating electrodes (Mödlin et al. 2005). Fibers further from the electrodes remain atrophied.

Another strategy to reduce atrophy after complete denervation has been to reinnervate muscle from replacement motoneurons. Not only will reinnervation restore the ability to excite muscle via nerve, regenerating axons potentially have access to all muscle fibers via intramuscular nerve sheaths. In animal models, neural progenitor cells, bone marrow stromal cells, and embryonic stem cells have been transplanted into the injured spinal cord to replace motoneurons (Deshpande et al. 2006; Lu et al. 2012; Mothe and Tator 2012; Reier 2004). The evidence for muscle reinnervation from these transplanted neurons is equivocal in neurologically complete injuries (Abematsu et al. 2010; Blesch and Tuszynski 2009; Wyatt et al. 2011). Functional benefits may have arisen from local sloughing of neurotrophic factors from the transplanted neurons that in turn stimulate host neurons to survive, produce axon sprouts, and release neurotransmitters (Abematsu et al. 2010; Bonner et al. 2011; Cummings et al. 2005; Jakeman and Reier 1991; White et al. 2010).

The axons of cells transplanted into the spinal cord also have a long distance to regenerate to reach muscle targets. Studies have shortened the distance between the transplanted cells and skeletal muscle by placing muscle near the spinal cord (Clowry et al. 1991). We have chosen to transplant embryonic neurons into peripheral nerve close to the target muscles. The muscles were reinnervated, but the evoked contractions were weak (Erb et al. 1993; Thomas et al. 2000). Both motoneuron survival and axon regeneration increased when the neurons were transplanted with a combination of neurotrophic factors or the transplant was stimulated electrically (20 Hz for 1 h; Casella et al. 2010; Grumbles et al. 2009, 2013), consistent with the neurotrophic and activity dependency of embryonic motoneurons (Brunet et al. 2007; Gallo et al. 1987; Goldberg et al. 2010).
2002; Hanson et al. 1998; Perez-Garcia et al. 2008). An important question is whether the additional axons that grow in response to brief transplant stimulation form motor units and improve muscle function. In this study, we have electrically stimulated embryonic motoneurons at various frequencies for up to 1 h immediately after cells were transplanted into the nerve. The transplant medium contained neurotrophic factors shown to improve motoneuron survival and axon growth (Grumbles et al. 2009). Our first aim was to determine whether brief electrical stimulation of the transplant increased the number of reinnervated motor units in ankle extensor muscles. Second, was stimulation frequency, pattern, duration, and/or the number of pulses most important for motor unit formation? Third, how do the contractile properties of the reinnervated medial gastrocnemius (MG) muscles compare to those of denervated and uninjured muscles? Functional behaviors can be restored to innervated muscles using patterned electrical stimulation whereas muscle denervation is an exclusion criterion for this approach.

MATERIALS AND METHODS

All procedures performed on animals were approved by the University of Miami Institutional Animal Care and Use Committee. These procedures adhered to the animal care and use guidelines of the National Institutes of Health. Ventral spinal cord cells dissociated from day 14 to 15 Fischer rat embryos were transplanted into 3-month-old inbred Fischer rats (mean weight: 163 ± 8 g; Harlan Laboratories, Indianapolis, IN) to avoid the need for immunosuppression. A total of 140 female rats were used in this study.

Experimental Design

The left sciatic nerve was transected to denervate many hindlimb muscles and to mimic the muscle consequences of motoneuron death. One week later, dissociated ventral spinal cord cells were purified for motoneurons and then injected into the left tibial nerve as the only neuron source for muscle reinnervation. The neurons were stimulated for up to 1 h, immediately after transplantation, to test whether this brief intervention altered the number of reinnervated motor units and contractile properties of ankle extensor muscles.

Muscle Denervation

Animals were anesthetized with an intraperitoneal injection of Nembutal (40 mg/kg ip; Akorn, Lake Forest, IL) for all surgeries and physiological recordings. Each animal was laid prone on a heating pad to keep body temperature at 37°C. The sciatic nerve was exposed to denervate muscles. Two sutures were tied to the sciatic nerve, ~4 mm apart, and ~15–20 mm proximal to where the tibial nerve entered the gastrocnemii muscles. A 2-mm section of nerve was removed between the sutures. These procedures produced denervation of multiple hindlimb muscles. The proximal end of the sciatic nerve was tied to the adductor femoris muscle and remained there until death, thereby preventing muscle reinnervation from axons of spinal motoneurons.

Embryonic Cell Preparation

Embryonic ventral spinal cord cells were dissociated and purified according to earlier methods (Camu and Henderson 1999). Briefly, the ventral spinal cord was dissected from day 14 to 15 embryos in calcium and magnesium-free PBS. The cord tissues were incubated in trypsin solution for 15 min, washed with Leibowitz-15 (L15) solution, and triturated in BSA using a fire-polished glass pipette. Ventral spinal cord cells were purified for motoneurons using a density gradient centrifugation technique (Taylor et al. 2007). The ventral spinal cells were suspended using Optiprep and L15 solution (Axis-Shield, Oslo, Norway) and centrifuged at 913 g for 15 min. Large cells were collected between the Optiprep and media (~73% motoneurons; Islet-1 was used to mark embryonic motoneurons; Grumbles et al. 2012), suspended in 4% BSA centrifuged for 10 min at 230 g, and resuspended in L15 solution. Neurotrophic factors were added to the medium of all cell transplants since they increased motoneuron survival in vitro (Hanson et al. 1998) and in our in vivo model (Grumbles et al. 2009). Factors included with the cells were brain-derived neurotrophic factor (BDNF; 10 ng/ml), ciliary neurotrophic factor (10 ng/ml), glial cell-derived neurotrophic factor (10 ng/ml), hepatocyte growth factor (10 ng/ml), insulin-like growth factor-1 (10 ng/ml), and forskolin (10 μM).

Cell Transplantation

Embryonic cells were transplanted into the tibial nerve of anesthetized animals 1 wk after denervation because a delay improved axon regeneration (Grumbles et al. 2002). A total of 200,000 purified ventral spinal cord cells in 5 μl medium were injected into the distal tibial nerve stump, 10–15 mm from where the tibial nerve entered gastrocnemius muscles. Cells were counted using Trypan blue staining and a hemocytometer. One group of animals only had 5 μl of medium injected into the tibial nerve without cells or neurotrophic factors to control for the effects of cell transplantation (No Cells group). We have previously demonstrated that injection of medium with neurotrophic factors but no cells does not result in muscle reinnervation (Casella et al. 2010).

Stimulation Intervention

Immediately after transplantation, the tibial nerve was laid on a pair of silver wire hook electrodes (0.65-mm diameter) to stimulate the neurons in the nerve (3-V, 100-μs duration pulses, charge-balanced square pulses, anode distal; Al-Majed et al. 2000b; Gustafsson and Jankowska 1976; S48 or S88 stimulator; Natus Neurology, Warwick, RI), which is supramaximal (~150%) for excitation of all axons in rat sciatic nerve. No electromyographic (EMG) was evoked in ankle extensor muscles in response to this stimulation because the sciatic nerve had been cut 1 wk earlier. The experiment involved eight groups of animals (Fig. 1). Animals in the High-Frequency group (n = 13) received continuous stimulation at 20 Hz for 1 h (n = 72,000 pulses) because this pattern of stimulation increased the number of motoneurons that initiated axon growth across the suture line following nerve transection (Al-Majed et al. 2000b). For this group, the maximum rest time (MRT) during the stimulation intervention was the same as the interpulse interval (50 ms). Animals in the Low-Frequency group (n = 13) received continuous stimulation at 1 Hz for 1 h (n = 3,600 pulses; MRT: 1,000 ms) because this stimulation sped up the return of motor function following nerve crush (Pockett and Galvin 1985). Animals in the Intermittent group (n = 13) received stimulation at 20 Hz for 6 s, every 2 min for 1 h (3,600 pulses; MRT: 114 s), a pattern of activity similar to that recorded from rat embryonic lumbar ventral roots (Nakayama et al. 1999). To control for stimulation frequency, animals in the Scrambled group (n = 12) received continuous stimulation for 1 h at various frequencies (11–30 Hz) that averaged 20 Hz but received the same number of pulses as the High-Frequency group (n = 72,000; MRT: 91 ms). This stimulation sequence was generated using a random number generator (random.org). To control for the duration of stimulation, animals in the Duration Control group (n = 13) received one 3-min block of stimulation at 20 Hz (3,600 pulses, the same number of pulses as the Low-Frequency and Intermittent groups; MRT: 57 min). For animals in the No Stimulation group (n = 12), the tibial nerve was laid on the electrodes for 1 h but no electrical
A pair of silver electrodes was laid on the mid-belly of each muscle to record EMG activity. The tibial nerve (transplant) was laid on another pair of silver electrodes for stimulation (SR88 stimulator). EMG and force were filtered (30–1,000 Hz and DC-100 Hz, respectively) and sampled online using a SC/Zoom system (3,200 and 400 Hz, respectively; Umeå University, Umeå, Sweden).

Experimental protocol for reinnervated and uninjured muscles. Each muscle was subjected to three types of stimulation. 1) Single pulses (30 V, 50 μs) were delivered to the transplant while increasing or decreasing the muscle length by 1 mm to determine the maximal twitch force. All subsequent stimulation was delivered at this optimal muscle length. 2) To count the number of reinnervated motor units, stimulation intensity (50-μs pulses) was increased from subthreshold (1 V) to supramaximal intensity (150 V) in 0.1-, 1-, or 10-V steps. Stimulation was stopped once maximum muscle force was observed consistently (no force increase with stronger stimulation). Two additional series of pulses were applied using different pulse durations (20 and 10 μs). 3) To determine muscle excitability, five single pulses of increasing intensity were delivered (10, 30, and 150 V each for 50 μs, 150 V for 100 μs, and 150 V for 1,000 μs).

Muscle contractile properties were examined only in MG because it has its own parent nerve, whereas the axons to LG and soleus travel in the same nerve branch. Contractions were evoked by stimulation at different frequencies (5, 8, 10, 15, 20, 30, 40, and 50 Hz for 1 s; 100 and 200 Hz for 0.5 s using 150-V and 50-μs pulses). Fatigue was induced by delivering 13 pulses at 40 Hz every second for 2 min (Burke et al. 1973). These data were compared with results from five uninjured muscles.

Stimulation of denervated muscles. Muscles in the No Cells group remained denervated because no EMG or force was evoked in response to transplant stimulation (150-V and 1,000-μs pulses). This denervation was confirmed by an absence of myelinated axons in the MG, LG, and tibial nerves (Liu et al. 2013). In these animals, the MG muscle was then stimulated directly to examine the excitability of denervated muscle using the same five pulses delivered to transplants. Stimulation at 40, 50, and 100 Hz was used to determine maximum force. Muscles were fatigued for 2 min, as for the transplants (150-V and 1,000-μs pulses).

Physiological Analysis

All analyses were completed offline using Zoom software.

Motor unit counts. The number of transplanted motoneurons that made functional connections with MG, LG, and PL muscles was estimated by counting the number of reinnervated motor units physiologically, as described previously (Thomas et al. 2003b). Three muscles were evaluated, rather than one muscle, to examine whether the stimulation intervention had general effects. For each muscle, all twitch forces were overlaid. Forces of similar magnitude were grouped and averaged. Mean force traces were ranked from weak to strong, and successive forces were digitally subtracted from each other to obtain the force of each motor unit. The number of force increments per muscle was taken as the number of reinnervated motor units. Motor unit force (baseline to peak force) was measured.

Whole muscle contractile properties. Twitch force (baseline to peak force), contraction time (CT; the time from force start to peak force), and half-relaxation time (hRT; time for the force to fall to half-maximal force) were measured. Multiple EMG potentials were regularly evoked when the transplant was stimulated at 150 V for 1,000 μs, but the number of responses and the force were inconsistent across transplants, so these data are not presented. The maximal force evoked in response to different stimulation frequencies (5–200 Hz) was measured. Force (relative to maximum) was plotted against frequency and used to calculate the frequency at which half-maximum force was achieved (F50) using linear regression (Thomas et al. 1991). The maximum force evoked at the start and end of the fatigue test was
measured and expressed as a ratio to provide the force fatigue index (final value/initial value). The fatigue index for EMG area was calculated similarly using measurements from the potential evoked by the initial pulse in the first and last trains of stimuli.

Myelinated axon numbers, muscle fiber size, and reinnervation. Following the physiological recordings, the nerves to MG, LG, and other tibial-innervated muscles distal to the MG and LG nerve branches were removed, fixed in 2% glutaraldehyde, embedded in Epon Araldite resin, sectioned (1 μm), and stained with Toluidine blue to visualize myelinated axons, as described previously (Liu et al. 2013). The number of myelinated axons in each MG nerve was counted (Metamorph software; Molecular Devices, Sunnyvale, CA) and compared with the number of MG motor units to determine the number of axons that successfully made functional connections with the reinnervated muscle fibers.

MG, LG, and PL muscles were also removed after the physiological recordings and frozen in isopentane cooled in dry ice. Cross sections (10 μm) of the MG muscles were cut, stained with hematoxylin and eosin (Electron Microscopy Science, Hatfield, PA) and imaged at ×40 magnification, and the areas of 500 sampled muscle fibers were measured (Metamorph software). The percentage of large muscle fibers (fiber cross-sectional area >498 μm²) was used to estimate the extent of muscle reinnervation. We have previously stimulated cell transplants repeatedly to deplete functional (reinnervated) muscle fibers of glycogen and determined that the glycogen-depleted (reinnervated) fibers had areas that were >95% of the fibers in completely denervated muscles (>498 μm²; Grumbles et al. 2008). Mean muscle fiber area and reinnervation were compared with the number of MG motor units to assess whether motor unit formation changed muscle atrophy and reinnervation, respectively.

Statistical Analyses

All statistical analyses were performed using SPSS Statistics 20 (IBM, Armonk, NY). Across group differences in the mean number of motor units, motor unit force, whole muscle twitch contraction time, half-relaxation time, F₅₀, force, and EMG fatigue indexes were analyzed using one-way ANOVA. The MG muscle force evoked at different stimulation frequencies (1–200 Hz) was compared across groups using two-way repeated-measures ANOVA. When analyses were significant (P < 0.05), post hoc tests (Tukey or Holm-Sidak) were performed. Pearson analysis was used to examine across-group relationships between the mean MG motor unit count and the myelinated axon count, reinnervated muscle fiber area, percentage of reinnervation, as well as maximal force and the maximum rest time and the mean motor unit count for MG, LG, and PL muscles. The count for MG, LG, and PL muscles was included for each animal so equal weight was given to each muscle. Means ± SD are given in the text and figures.

RESULTS

Continuous Transplant Stimulation for 1 h Increased Motor Unit Numbers

Stimulation of the transplants at increasing intensities resulted in varying numbers of force increments, each step representing the recruitment of another motor unit. The number of motor units varied across muscles (17, 7, and 2 units in Fig. 2, A–C). In reinnervated MG, LG, and PL muscles, motor unit counts ranged from 0–25, 0–21, and 0–16, respectively (n = 76 for each muscle). Table 1 shows motor unit counts by muscle and group and includes estimates for uninjured animals. The force, CT, and hRT of different motor units also varied within and across muscles.

![Fig. 2. Motor unit counts and force. Overlay of averaged medial gastrocnemius (MG) force steps evoked by stimulation of a transplant from the High-Frequency (A), Scrambled (B), and Duration Control (C) groups 10 wk after cell transplantation. D: mean MG, lateral gastrocnemius (LG), and plantaris (PL) motor unit count across groups vs. maximum rest time (log₁₀ scale, R² = 0.992; Pearson correlation). Data from the No stimulation group were excluded from the correlation. E: mean (±SD) motor unit force of MG, LG, and PL muscles across groups, expressed relative to the respective uninjured whole muscle twitch forces. Short group names are used in all figures: No Stim, No Stimulation; Dur, Duration Control; Int, Intermittent; Low, Low-Frequency; Scr, Scrambled; High, High-Frequency.](http://jn.physiology.org/)
similar to the number of motor units in the No Stimulation group (60-min rest). Unit counts and MRT were intermediate for animals in the Intermittent and Low-Frequency groups. These data suggest that transplant stimulation for 1 h with shorter MRT was more important for motor unit formation than the frequency of stimulation or the number of pulses. Both the Duration Control and High-Frequency groups received 20-Hz stimulation, but there were significantly more motor units in High-Frequency group ($P = 0.014$). Both the Duration Control and Low-frequency groups received 3,600 pulses, but there were more motor units in the Low-Frequency group ($P = 0.038$). In groups that received 1 h of stimulation with short rests (High, Scrambled, and Low), the motor unit count exceeded that for the Duration Control group ($P = 0.004$) and No Stimulation group ($P = 0.045$).

Mean (±SD) motor unit force for MG, LG, and PL muscles was similar across groups (range: 0.79 ± 0.44 to 1.12 ± 0.51% of uninjured muscle twitch force; for MG: 4.0 ± 4.8 to 7.7 ± 4.6 mN), irrespective of whether the cells were stimulated immediately after transplantation or not (Fig. 2E).

Transplant Stimulation Improved Motor Unit Counts, Muscle Reinnervation, and Force

Higher motor unit counts resulted in greater muscle reinnervation ($P = 0.041$; Fig. 3A) and stronger maximal tetanic forces ($P = 0.021$; Fig. 3B). Although not significant, higher motor unit counts were associated with greater numbers of axons in the MG nerve ($P = 0.098$) and larger muscle fiber areas ($P = 0.072$).

Reinnervated Muscles Reached Half-Maximal Force at Low-Stimulation Frequencies

Force fusion was more prominent at low-stimulation frequencies in reinnervated (e.g., 8 or 20 Hz; Fig. 4A) vs. uninjured muscles (Fig. 4B). When responses to different frequencies of stimulation were normalized to maximal muscle force, there were differences by group ($P < 0.001$), frequency ($P < 0.001$), and an interaction between group and frequency ($P < 0.001$). All groups of reinnervated muscles generated higher relative forces than uninjured muscles between 15 and 50 Hz ($P < 0.05$; Fig. 4C). Half-maximal force ($F_{50}$) differed by group ($P < 0.001$) and was reached at significantly lower frequencies for all reinnervated muscle groups (ranging from 15 ± 5 Hz for the Duration Control group to 21 ± 9 Hz for the Low-frequency group) vs. uninjured muscles ($42 ± 4 Hz, P < 0.001$). Within reinnervated muscle groups, the $F_{50}$ for the Duration Control group differed from the Low-, High-, and No-Stimulation groups ($P \leq 0.02$) and the Scrambled and Intermittent groups differed from the Low-Frequency group ($P \leq 0.03$).

Twitch contraction times ($P = 0.001$) and half-relaxation times ($P = 0.009$) differed by group. All groups of reinnervated muscles had slower twitch contraction times and half-relaxation times than uninjured muscles (Fig. 4, D and E); both $P \leq 0.004$; CT: from 31 ± 5 to 42 ± 9 ms across groups; within reinnervated muscle groups only the Scrambled group was different from the Low-Frequency group, $P = 0.03$; hRT: from 48 ± 19 to 57 ± 19 ms across groups), one factor that contributed to the greater fusion of forces in response to lower stimulation frequencies.

Table 1. Motor unit counts by muscle

<table>
<thead>
<tr>
<th>Group</th>
<th>MG, n</th>
<th>LG, n</th>
<th>PL, n</th>
<th>Total, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stimulation</td>
<td>3 ± 4 (0–12)</td>
<td>3 ± 3 (0–9)</td>
<td>3 ± 4 (0–11)</td>
<td>10 ± 8 (0–22)</td>
</tr>
<tr>
<td>Duration Control</td>
<td>1 ± 2 (0–6)</td>
<td>1 ± 2 (0–5)</td>
<td>4 ± 5 (0–13)</td>
<td>7 ± 7 (0–23)</td>
</tr>
<tr>
<td>Intermittent</td>
<td>4 ± 3 (0–9)</td>
<td>3 ± 3 (0–12)</td>
<td>4 ± 3 (0–11)</td>
<td>10 ± 7 (0–22)</td>
</tr>
<tr>
<td>Low Frequency</td>
<td>5 ± 8 (0–24)</td>
<td>3 ± 3 (0–8)</td>
<td>5 ± 5 (0–16)</td>
<td>13 ± 11 (0–35)</td>
</tr>
<tr>
<td>High Frequency</td>
<td>5 ± 5 (0–17)</td>
<td>5 ± 5 (0–12)</td>
<td>5 ± 4 (0–13)</td>
<td>15 ± 12 (0–35)</td>
</tr>
<tr>
<td>Scrambled</td>
<td>5 ± 7 (0–25)</td>
<td>6 ± 6 (0–21)</td>
<td>4 ± 3 (0–10)</td>
<td>15 ± 13 (1–49)</td>
</tr>
<tr>
<td>Uninjured</td>
<td>82 ± 3 (78–87)</td>
<td>106 ± 8 (93–115)</td>
<td>——</td>
<td>——</td>
</tr>
</tbody>
</table>

Values are means ± SD (range). Data are from 12 or 13 animals per group. Uninjured medial gastrocnemius (MG) and lateral gastrocnemius (LG) data are from Grumbles et al. (2002), adjusted to include fibers >6 μm and then halved to account for sensory axons; plantaris (PL) data are from Pettigrew and Gardiner (1987).

Fig. 3. Correlations between parameters. Mean MG motor unit count as a function of the percentage of reinnervated muscle fibers (A; $R^2 = 0.69$) and maximum muscle tetanic force (B; $R^2 = 0.77$, Pearson correlations).
When trains of pulses were delivered at 40 Hz for 2 min, the evoked force decline differed by group ($P < 0.001$) and was less in reinnervated (Fig. 5A) than uninjured muscles (Fig. 5B). The mean force fatigue indexes ranged from $0.53 \pm 0.19$ (Duration Control group) to $0.84 \pm 0.07$ (High-Frequency group) for reinnervated muscles, all of which were significantly higher than the mean for uninjured muscles ($0.23 \pm 0.07$; $P < 0.001$; Fig. 5C). Within reinnervated muscles, only the Duration Control and High-Frequency groups differed ($P < 0.05$). Fatigue indexes for EMG area did not differ for reinnervated (range: $1.09 \pm 0.25$ to $1.28 \pm 0.22$ across groups) and uninjured muscles ($1.01 \pm 0.35$; Fig. 5C) or within reinnervated groups. These results suggest there was effective transmission of signals to the sarcolemma in reinnervated and uninjured muscles and that the force declines must reflect impaired processes in the muscle fibers.

**Denervated Muscles Only Generated Force Through Direct Muscle Stimulation**

Stimulation of the tibial nerve in the No Cells group resulted in no evoked EMG or force at any stimulus intensity or frequency. The nerves to the MG, LG, and distal tibial muscles in these animals also had no myelinated axons (Liu et al. 2013). Thus these muscles remained denervated. The only way to elicit force was to stimulate the denervated muscles directly (Fig. 6). Direct muscle stimulation at 10, 30, and 150 V (50-μs pulse duration) generated an average twitch force of $0.5\% (2.2 \pm 2.1 \text{ mN})$ compared with $36.9 \pm 45.8 \text{ mN}$ in reinnervated muscles, 18% and 88% maximal, respectiv
Results suggest that patterned electrical stimulation of embryonic neurons, in vivo, has long-term functional consequences across muscles.

**Electrical Stimulation for 1 h with Short Rests Increased Motor Unit Numbers**

Neurons isolated from developing spinal cord by trypsin digestion and triturations are relatively free of dendrites, axons, trophic interactions, and synaptic communications. Most of these neurons die in vitro and after transplantation, so they are vulnerable. Although the principal route to promote survival of motoneurons has been to add growth factors (Casella et al. 2010; Grumbles et al. 2009; Henson et al. 1998; Kaal et al. 1997; Reier 2004; Tetzlaff et al. 2011), freshly dissociated neurons do display functional ion channels and pumps (e.g., calcium, sodium, and Na\(^+\)/K\(^-\) pump) that are responsible for spontaneous action potentials in the absence of synaptic inputs (Dobretsov et al. 1999; Raman et al. 2000; Swensen and Bean 2003). It is the retention of these various channels that is critical for activity-dependent neuron survival and axon growth (Subramanian et al. 2012).

Electrical stimulation of neurons may activate several signaling pathways (Lyons and West 2011) and increase reinnervated motor unit numbers by enhancing gene transcription. In vitro, promotion of retinal ganglion cell survival and axon growth by electrical stimulation depended on sodium channel expression and upregulation of cAMP (Goldberg et al. 2002). Upregulation of cAMP also increased the number of embryonic motoneurons that extended neurites and the length of the neurites (Aglah et al. 2008). These observations extend earlier findings where stimulation enhanced growth cone motility though phosphorylation of nuclear Ca\(^{2+}\)/cAMP-response element binding protein (CREB; Anglister et al. 1982; Bito et al. 1996; Liu and Graybiel 1996). A principal target of CREB in many kinds of neurons is the BDNF gene promoter (Lyons and West 2011). If synthesized by our transplanted neurons, BDNF may promote the differentiation of motoneurons and motor axon growth (Naem et al. 2002; Zurn et al. 1996), one possible mechanism to explain the stimulation-specific increase in motor unit counts (Fig. 2D). Other studies show that electrical stimulation of the proximal stump of a transected peripheral nerve increased the number of motor axons that grew across the suture line and sped up the return of muscle function (Al-Majed et al. 2000b; Pockett and Gavin 1985) by increasing BDNF expression in neurons (Al-Majed et al.
Electrical stimulation also induced motoneuron-specific enhancement of HNK-1 expression, a downstream target of BDNF, and this correlated with accelerated muscle reinnervation (Eberhardt et al. 2006). In contrast, a Cre-induced conditional knockout of BDNF in Schwann cells decreased axon regeneration after nerve transection and repair in mice. In addition, a tibial nerve graft from these same animals did not support axon regeneration in mice with a subset of neurons that lacked BDNF (Wihelm et al. 2012).

In vitro studies show that activity-dependent regulation of CREB phosphorylation is dependent on the temporal pattern and duration of signaling. Stimulation for at least 30 min was required for sustained levels of phosphorylated CREB and for decreased dephosphorylation by phosphatase-1 (Bito et al. 1996; Liu and Graybiel 1996; Lyons and West 2011). Our results show continuous stimulation of transplanted neurons for 1 h increased the number of reinnervated motor units beyond that measured after 3 min of stimulation (Fig. 2D). Second, others have shown that longer rests between trains of stimuli resulted in lower levels of phosphorylated CREB and immediate early gene (cfos) expression (Fields et al. 1997). We had higher reinnervated motor unit numbers in groups where the stimulation protocol included shorter rest times (Fig. 2D). Together, our data support the importance of the pattern of pulses and stimulation duration in motor unit formation. Stimulation frequency or the number of pulses delivered was less critical.

Higher Motor Unit Counts Enhance Muscle Reinnervation and Force

Groups with more reinnervated MG motor units had more reinnervated muscle fibers (Fig. 3A) and stronger muscles (Fig. 3B) without significant group differences in muscle fiber size (Liu et al. 2013) or motor unit force (Fig. 2E). These results suggest that the ability to form more functional motor units was the main long-term effect of electrical stimulation. Brief electrical stimulation may also induce regeneration of more axons from similar kinds of motoneurons. Most motoneurons that survived in the transplants were small. Mean diameter across groups ranged from 21.2 ± 1.2 to 23.0 ± 1.2 μm (Liu et al. 2013). As a consequence, our reinnervated muscles were weak, slow, and fatigue resistant, with few within group differences (Figs. 3B, 4, and 5). The Duration Control group differed most in its contractile properties, suggesting that short duration stimulation of embryonic neurons may be detrimental. Overall, the reinnervated muscle properties were characteristic of fatigue resistant motor units (Botterman et al. 1985; Kernell et al. 1983) and consistent with the predominance of type IIA and I fibers in muscles reinnervated from embryonic motoneurons (Thomas et al. 2003b). These results also support the idea that motoneurons have a strong influence over the contractile properties of the muscle fibers they reinnervate (Buller et al. 1960; Dum et al. 1985a,b; Gordon et al. 1986; Foehring et al. 1987).

When embryonic stem cells were transplanted into peripheral nerve in mice (Yohn et al. 2008), the number of reinnervated motor units was higher. However, the motor units and muscles were very weak compared with the wild-type mice, and the muscles were fatigable (Diaz et al. 2005; Hege dus et al. 2007; Manuel and Heckman 2011).

Contractile properties are often restored when muscles are reinnervated from spinal motoneurons provided there is no delay in nerve repair (Gordon and Stein 1982; Hennig and Lømo 1987; Kobayashi et al. 1997). In our case, muscle weakness arose from reinnervation of only some fibers (Fig. 3A). Axons from noncholinergic neurons present in our transplants may fail to innervate muscle (Grumbles et al. 2012). The neuromuscular junctions do function reliably, however, as shown by the maintenance of EMG area in response to repeated stimulation (Fig. 5C). In addition, EMG amplitude did not change markedly when stimulation was delivered at different frequencies. These results suggest that subthreshold innervation (Ijkema-Paassen et al. 2001; McArdle 1975) was not a major factor 10 wk after cell transplantation. Nevertheless, some junctions may be immature and nonfunctional, in part because the muscles are inactive (Grumbles et al. 2012; Sanes and Lichtman 1999). Without muscle activity and loading, atrophy and reductions in strength are significant (Pierrot et al. 1991). Increases in CT and hRT with reinnervation (Fig. 4E) may arise from reduced use, possibly from slowing of calcium uptake (Duchateau and Hainaut 1987; Howell et al. 1997), from increases in the proportion of type I and IIA fibers (Foehring et al. 1986; Thomas et al. 2003b), and/or from greater muscle compliance after reinnervation (Huyghues-Despointes et al. 2003). Both the slow CT and hRT, as well as the higher twitch-tetanic force ratios, contributed to the greater force fusion at a given stimulation frequency and achievement of half-maximal force at 15 ± 2 to 21 ± 3 vs. 42 ± 2 Hz in reinnervated vs. uninjured muscles, respectively (Fig. 7B).

Muscle Innervation Is Needed To Restore Function with Patterned Electrical Stimulation

After denervation, muscles became weak, slow, fatigable, and less responsive to electrical stimulation (Fig. 6), confirming previous data (e.g., Ashley et al. 2007; Finol et al. 1981; Gundersen 1985; Gutmann and Young 1944; Kotsias and Muchink 1987; Schmalbruch et al. 1991). Denervated muscles could only be excited by direct muscle stimulation. Not only was little force evoked in denervated muscles by direct stimulation at low intensities, one high intensity, wide duration pulse evoked 66 ± 1% of maximal force (Fig. 7A). This leaves limited scope to grade force by changing pulse width, intensity, or frequency. Denervated muscles were also fatigable (Fig. 7C). These less than optimal contractile properties illustrate why denervation is an exclusion criterion for functional electrical stimulation applications. The high-stimulation intensities needed to excite denervated muscles would also raise the power requirements of any stimulation device and may induce tissue damage, and current spread would make selective muscle activation difficult.

Reinnervation of muscle from embryonic neurons offers multiple advantages for producing functional movements by patterned electrical stimulation, an approach used to generate behaviors such as pinch and key grips in people with high cervical spinal cord injury allowing them to eat independently (Peckham and Knutson 2005). Maximal twitch forces were gradable at relatively low-stimulation intensities by changing pulse amplitude or duration because nerve is more excitable than muscle (Mortimer 1981). Recruitment of all axons by a single stimulus evoked 26 ± 2% of maximal force (Fig. 7A),
leaving an average of 74% of force to be generated by modulating stimulation frequency (Bottermann et al. 1986). Further, half-maximal muscle force was reached at 17 ± 1 Hz (Fig. 7B). Use of fewer pulses may reduce fatigue (Garland et al. 1988; Thomas et al. 2003a). Even though reinnervated MG muscles often produced a fraction of the force of uninjured muscles (~5% uninjured, on average, in groups receiving 1 h of stimulation with short MRT; Fig. 3B), other synergists extend the ankle. Together, MG, LG, and PL contained up to 49 motor units (Table 1). When all of these synergists are activated together by transplant stimulation, these muscles are likely to produce more than enough force for locomotion (~5% maximal; Ichihara et al. 2009; Jung et al. 2009; Thomas et al. 2010) or to move a joint through its range against gravity (9% force; Needham-Shropshire et al. 1997). The fatigue resistance of reinnervated muscles (Fig. 7C) also allows them to contract at a given intensity for a long period of time. Further, the main effect of brief electrical stimulation on the transplanted neurons was the formation of more motor units (Fig. 2D). Higher motor unit numbers increase the number of axons to stimulate, the potential to grade force finely, and force production. Reinnervation of muscle from neurons transplanted in a nearby peripheral nerve may also be a strategy to arrest muscle atrophy in other situations involving chronic muscle denervation. After proximal nerve injuries, peripheral axons often have to regenerate long distances to reach muscle targets. The delay in muscle reinnervation can induce severe muscle atrophy. Early reinnervation from replacement neurons may result in a less atrophied muscle, which could facilitate subsequent reinnervation from regenerating peripheral axons.

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AUTHOR CONTRIBUTIONS


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