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Effects of Brief Electrical Stimulation on Embryonic Neurons Transplanted into Peripheral Nerve

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EFFECTS OF BRIEF ELECTRICAL STIMULATION ON EMBRYONIC NEURONS TRANSPLANTED INTO PERIPHERAL NERVE

By

Yang Liu

A DISSERTATION

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of the University of Miami
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EFFECTS OF BRIEF ELECTRICAL STIMULATION ON EMBRYONIC NEURONS TRANSPLANTED INTO PERIPHERAL NERVE

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Motoneuron death occurs with spinal cord injury, neuromuscular disease and age, resulting in muscle denervation. Although spared axons can reinnervate nearby muscle fibers, death of an entire motoneuron pool prevents muscle reinnervation by host motoneurons. The muscle will remain denervated completely. To reduce denervation-induced muscle atrophy, we have previously transplanted embryonic ventral spinal cord cells into a peripheral nerve near denervated muscles. This strategy shortens the distance axons have to regenerate to reinnervate muscles. Reinnervation reduced muscle atrophy but some muscle fibers remained denervated. Since neural activity is required for the survival of embryonic neurons and promotes axon growth, the aim of this study was to test whether brief electrical stimulation of embryonic neurons transplanted into peripheral nerve changes motoneuron survival, axon regeneration, motor unit formation and muscle properties 10 weeks after transplantation. The sciatic nerve was transected to induce muscle denervation, thereby mimicking the muscle consequences of motoneuron death. One week later, 200,000 embryonic day 14-15 ventral spinal cord cells, purified for motoneurons, were injected into the tibial nerve of adult Fischer rats close to gastrocnemii muscles. The transplanted neurons were the only source of neurons for muscle reinnervation because the proximal stump of the sciatic nerve had been tied to a
hip muscle. Immediately after transplantation, the cells were stimulated for up to one hour using various protocols designed to determine the differential effects of pulse number, stimulation frequency, pattern and duration of stimulation on outcomes. Low frequency stimulation (1 Hz) for one hour resulted in more myelinated axons in the tibial nerve compared to intermittent stimulation, 3 minutes of stimulation or no stimulation. Continuous stimulation for one hour resulted in higher numbers of reinnervated motor units than 3 minutes of stimulation or no stimulation. Higher motor unit numbers resulted when the stimulation intervention included shorter rest times. Muscles with higher motor unit counts were stronger. Even without electrical stimulation, cell transplantation itself and reinnervation reduced muscle atrophy significantly. Muscles reinnervated by embryonic motoneurons could be excited by nerve stimulation whereas denervated muscles were unresponsive. Higher forces, relative to maximum, were attained in response to 15-50 Hz stimulation in reinnervated versus uninjured muscles. Reinnervated muscles were resistance to fatigue. The ability of embryonic neurons to respond to brief electrical stimulation, in vivo, by forming more motor units makes these reinnervated muscles more useful for production of functionally meaningful movements using patterned electrical stimulation.
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Chapter 1

Introduction

Certain diseases, such as amyotrophic lateral sclerosis or poliomyelitis, and trauma to the spinal cord, kill motoneurons and thereby denervate muscles (Sharrard 1955; Tandan and Bradley, 1983; Thomas, 1997). When some motoneurons in a motor pool die, intact axons sprout within the muscle to restore innervation (Thompson and Jansen, 1977). However, death of all of the motoneurons within a pool denervates the muscle completely, causing rapid and progressive atrophy (Degens et al., 2008; Finkelstein et al., 1993). Chronic stimulation of denervated muscles reduces atrophy (Hennig and Lømo, 1987; Salmons and Vrbová, 1969; Westgaard and Lømo, 1988), but effects are often local (Mödlin et al., 2005). Another approach to arrest denervation atrophy is to restore muscle innervation by replacing motoneurons. Neural progenitor cells, bone marrow stromal cells, and embryonic stem cells have been transplanted into the spinal cord as substitute motoneurons. In general, neuron survival is low (Abematsu et al., 2010; Deshpande et al., 2006; Reier, 2004; Sandner et al., 2012; Wyatt et al., 2011). Nerves and muscles also deteriorate with time. To minimize the time available for muscle atrophy after denervation, a local motoneuron replacement strategy was developed. Ventral spinal cord cells isolated from rat embryos were transplanted into the tibial nerve of adult rats near the denervated muscles. Axons had to grow only a short distance from the transplanted neurons to reinnervate muscles, which reduced atrophy. The reinnervated
muscles generated force in response to stimulation of the transplant but the contractions were weak (Thomas et al., 2000).

Central to complete recovery of muscle function is survival of the transplanted motoneurons, growth of axons from the cells, and formation of functional neuromuscular junctions. Embryonic neurons depend on neurotrophic factors and activity for survival. In addition, survival of neurons does not guarantee that they will grow axons (Brunet et al., 2007; Gallo et al., 1987; Goldberg et al., 2002; Hanson et al., 1998; Perez-Garcia et al., 2008). In this thesis I ask whether brief electrical stimulation of the transplanted neurons promotes motoneuron survival and axon regeneration (Chapter 3). Further, does this stimulation treatment influence the number of functional connections with muscle? If so, do the effects depend on the number of pulses delivered? Is the frequency of stimulation, its pattern and duration also relevant (Chapter 4)? Literature that forms the basis of these ideas is reviewed in Chapter 2. Chapter 5 presents our conclusions and a brief discussion of the implications for muscle function. Muscle denervation is an exclusion criterion for using patterned electrical stimulation to restore muscle function (Peckham and Knutson, 2005). Thus, interventions that restore muscle reinnervation and improve force generating capacity are of practical and clinical importance.
2.1 Motoneuron death is common

Motoneuron death occurs with trauma, neurological disease and age. Physical impact to the spinal cord induces motoneuron death within hours of injury. The extent of secondary neuron death that occurs during the weeks and months post injury is more controversial (Hulsebosch, 2002). This damage may occur over several spinal segments and eliminate complete motoneuron pools (Bunge et al., 1997; Kakulas, 2004; Milhorat et al., 1995). For example, some muscles supplied by the lesion epicenter are unresponsive to electrical stimulation of their peripheral nerves in chronic SCI, indicating complete denervation (Bryden et al., 2004; Kern et al., 2004; Mulcahey et al., 1999; Peckham et al., 1976; Thomas 1997; Thomas and Zijdewind, 2006).

Progressive motoneuron death occurs in people with amyotrophic lateral sclerosis, indicated by a decrease in the number of motor units in a muscle (Gooch and Harati, 2000; Olney et al., 2000; Ridall et al., 2006). Life expectancy is often only 3-5 years after diagnosis (Bradley et al., 1983; Rowland and Shneider, 2001). In people with spinal muscular atrophy, motoneuron death induced by genetic mutation can result in early childhood death in severe cases, but some may live in wheelchairs with normal life expectancy (Fidziańska and Rafałowska, 2002). People with poliomyelitis experience early motoneuron death from a viral infection. A second wave of motoneuron death may occur later in life and increase the amount of muscle paralysis (Farbu et al., 2006;
Motoneuron death also occurs as part of natural aging, beginning at about 60 years (Campbell et al., 1973; Doherty et al., 1993). At present, there is no effective method to restore muscle function after extensive motoneuron death (Blesch and Tuszynski, 2009; Gowing and Svendsen, 2011; Lindvall et al., 2012). When there are no host motoneurons available for muscle reinnervation, replacement of motoneurons by cell transplantation is one strategy which may lead to restoration of muscle function. This study addresses complete muscle denervation, which may occur after extensive motoneuron death. The focus is on replacement of motoneurons to restore muscle innervation.

### 2.2 Transplanted cells must survive if they are to improve muscle function

Different types of cells have been transplanted at various locations to replace motoneurons. For example, embryonic stem cells, adult neural stem cells and neural progenitor cells have been transplanted into the injured spinal cord or peripheral nerves as replacement motoneurons (Clowry et al., 1991; Erb et al., 1993; Gao et al., 2005; Howland et al., 1995; Lu et al., 2012; McDonald et al., 1999; Stokes and Reier, 1992; Thomas et al., 2000; Yohn et al., 2008). Most cells die within 24-48 hours of transplantation, regardless of the cell type or the transplant location (Barker et al., 1996; Emgård et al., 1999; Sortwell et al., 2000). Survival of transplanted cells rarely exceeds 10% (Deshpande et al., 2006; Grumbles et al., 2002, 2012; Wyatt et al., 2011). These findings make enhancement of neuron survival an important goal in cell transplantation studies. Here, I test how well brief electrical stimulation of transplanted neurons improves motoneuron survival (Chapter 3).
2.3 Embryonic ventral spinal cord as a source of motoneurons for transplantation

Neurogenesis in the rat spinal cord follows a ventral to dorsal pattern. Using $[^{3}H]$thymidine to label proliferating cells, Nornes and Das (1972) showed that developing neurons first appear at the ventral region of the rat spinal cord on embryonic day 11 (E11). These neurons are motoneurons, have large diameters (>30 µm), and continue to be born until E13. Sensory neurons and interneurons are born later. Further, there is a rostro-caudal pattern of neurogenesis along the spinal cord, with birth of lumbar neurons lagging behind cervical neurons by about one day.

Approximately 50 % of motoneurons undergo programmed cell death during development, starting at E13.5-16.5 in rats (Yamamoto and Henderson, 1999). Studies suggest that motoneurons have an intrinsic death clock that instructs them to die around the time of target innervation (E13) if neurotrophin levels are insufficient (Oppenheim, 1991; Sedel et al., 1999). Extrinsic factors may also influence developmental cell death. The death receptor Fas and its ligand FasL are expressed around the time of programmed cell death and are down regulated by neurotrophic factors (Raoul et al., 1999). Tumor necrosis factor α, secreted by macrophages in the somite, can also instruct motoneurons to die at a later time after target reinnervation (Sedel et al., 2004).

The percentage of motoneurons is highest at E13-15, reflecting a balance between birth and programmed cell death (Altman and Bayer, 1984; Oppenheim, 1986). Embryonic motoneurons have innervated muscles by E14-15. It was previously determined that E14-15 ventral spinal cord cells innervate adult rat muscle fibers better than younger cells (Grumbles et al., 2005), so E14-15 cells were transplanted here.
However, dissociation disrupts muscle-derived trophic support to embryonic motoneurons, making them vulnerable to die.

### 2.4 Trophic factors are required for motoneuron survival and axon regeneration

Embryonic motoneurons depend on several kinds of trophic factors for survival. These factors arise from different sources. For example, the survival of motoneurons can be promoted by trophic factors derived from muscle, including hepatocyte growth factor (HGF), cardiotrophin-1 (CT-1) and brain-derived neurotrophic factor (BDNF) (Arce et al., 1998; Jung et al., 1994; Pennica et al., 1996; Sendtner et al., 1992). Motoneuron survival also depends on Schwann cell derived trophic factors, including insulin-like growth factor-1 (IGF-1), ciliary-derived neurotrophic factor (CNTF), neurotrophin-3 (NT-3) and glial cell-derived neurotrophic factor (GDNF) (Arakawa et al., 1990; Henderson et al., 1993; Neff et al., 1993; Yan et al., 1992). Neurotrophic factors can have specific and overlapping roles. For example, GDNF promotes survival of most motoneurons, whereas HGF specifically increases the survival of lumbar but not cervical motoneurons (Arce et al., 1998; Henderson et al., 1994; Oppenheim et al., 1995; Novak et al., 2000; Yamamoto et al., 1997).

Not surprisingly, neural transplants require multiple trophic factors for survival and axon growth. Hanson and colleagues (1998), for example, increased the survival of motoneurons for 3 weeks in culture using a combination strategy that elevated the level of cAMP and BDNF, CNTF, GDNF, HGF, and IGF-1. In our model, the addition of single neurotrophic factors into the medium containing the cells had no significant effect on motoneuron survival or axon growth. However, when GDNF, HGF, IGF-1 and
forskolin were included in the medium with the embryonic cells, the numbers of ChAT-positive neurons and myelinated axons were higher, leading to greater muscle fiber reinnervation and stronger motor unit forces (Casella et al., 2010; Grumbles et al., 2009). This same combination of factors is included in our cell transplant medium here to promote neuron survival and axon growth.

2.5 Motoneuron survival is activity-dependent

Membrane depolarization induced using a potassium bath ([K⁺]) or electrical stimulation can improve embryonic motoneuron survival (Brunet et al., 2007; Gallo et al., 1987; Goldberg et al., 2002; Perez-Garcia et al., 2008). These effects are mediated through many channels on the neuron membrane and may involve multiple pathways. For example, calcium influx through L-type voltage-gated calcium channels promotes neuron survival by elevating cAMP levels and activating adenylate cyclase (Collins and Lile, 1989; Cooper et al., 1995; D’Mello et al., 1993; Flavell and Greenberg, 2008; Gallo et al., 1987; Koike et al., 1989; Meyer-Franke et al., 1995; Rydel and Greene, 1988) or by binding to calmodulin dependent protein kinases (Hack et al., 1993; Hanson and Schulman, 1992). The activation of these pathways in turn results in the phosphorylation of transcription factors such as cAMP-responsive element binding protein (CREB) and translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to increase the transcription of anti-apoptotic genes like bcl-2 (Goldberg et al., 2002; Polleux and Snider, 2010; Zheng and Poo, 2007). Elevated expression of anti-apoptotic proteins prevents cell death. In addition, electrical stimulation may activate Ras/Raf and phosphatidylinositol-3-OH kinase (PI3K) pathways to promote neuron survival (Bonni et
al., 1999; Dudek et al., 1997; Lev et al., 1995; Miller et al., 1997; Rosen et al., 1994; Xia et al., 1995; Yao et al., 1995). Other types of channels and pumps, including P-type and T-type calcium channels, sodium channels, large-conductance potassium channels and Na\(^+\)/K\(^+\) pumps, are also expressed by dissociated neurons. Activation of some of these channels and pumps underlies the spontaneous action potentials generated in the absence of synaptic inputs (Dobretsov et al., 1999; Raman et al., 2000; Swensen and Bean, 2003) and this may promote neuron survival. Further, dissociation can damage the neuron membrane. Calcium influx induced by electrical stimulation can reseal damaged membranes and maintain ionic balance, which is necessary to prevent cell death, to conduct action potentials, and to produce growth cones (Bloom and Morgan, 2011; Kamber et al., 2009; Spira et al., 2003; Xie and Barrett, 1991). Here, I test if dissociated embryonic motoneurons transplanted into peripheral nerve respond to brief electrical stimulation \textit{in vivo}, and cause long-term effects on muscle reinnervation.

When embryonic spinal neurons are isolated by trypsin digestion and trituration, they are relatively free of dendrites, axons, trophic interactions and synaptic communications (Hanson et al. 1998; Grumbles et al. 2009). Any remaining motor axons are likely damaged during the process of dissociation (Schnaar and Schaffner, 1981). Thus, electrical stimulation of transplanted embryonic motoneurons probably occurs by activation of ion channels present in the membrane of the neuronal cell bodies (Dobretsov et al. 1999; Raman et al. 2000; Swensen and Bean, 2003; Walton et al., 1993). If the initial segment of neurites has regenerated by the time of the stimulation intervention, which can occur 1-3 hours after dissociation (Bateille et al., 1998), the
pulses may excite these axons because the axon hillock can be more excitable than the cell body when the current source is further from the excitable tissues (Eccles, 1957).

Excitation of neurons or axons depends on the amount of charge injected into the nerve in response to the electrical stimulation. Assuming that the resistance of the rat sciatic nerve is similar to the frog sciatic nerve (478 Ω; Weerasuriya et al., 1984), the current injected into the sciatic nerve during brief electrical stimulation is 6.28 mA (I = V/R: 3 V / 478 Ω). The minimum threshold for exciting a single neuron or axon is 200-300 µA at 1.5 mm from the neuron or axon (Gustafsson and Jankowska, 1976), which is much lower than the current injected into the sciatic nerve in our transplant model. Therefore, the voltage delivered during the stimulation intervention is likely to be more than enough to excite both neurons and/or the initial segment of newly regenerated neurites in the transplant.

The voltage required to excite axons 10 weeks after cell transplantation is higher than that of uninjured axons (2.3-2.5V) because regenerated axons are smaller in diameter (Liu et al., 2013), and because connective tissue formation can increase the impedance of nerve tissue. By increasing the voltage, a similar amount of charge can be injected into the nerve to excite the regenerated axons. In addition, use of a constant current stimulator would ensure that the same amount of charge was injected into the nerve even if the impedance changed. Thus, the amount of charge delivered, rather than the voltage, determines whether axons will be excited.
2.6 Spontaneous activity is common during development

Spontaneous movements are observed early during development including in animals and humans (Narayanan et al., 1971; Felt et al., 2012). In rodents, repeated single potentials can be recorded from ventral roots 2 days after motoneuron birth, around E13-14 (O’Donovan and Landmesser, 1987; Saito, 1979). At E14-15, cervical and lumbar motoneurons produce spontaneous bursts of activity every 2.1±0.3 minutes that last 4.6±1.5 seconds, even after midthoracic spinal cord transection (Nakayama et al., 1999). As the spinal cord develops and neuromuscular junctions are formed, activity consists of the more complex multiple potentials (around E16-17; Dennis, 1981; O’Donovan et al., 1998; Saito, 1979). This spontaneous activity arises from cholinergic, GABAergic and glycinergic inputs and is often rhythmic, suggesting it may be important for the formation of neuronal networks (Hanson and Landmesser, 2003; Nishimaru et al., 1996). In this study, the transplants in one group of animals were stimulated with an intermittent protocol that is similar to the neuronal activity recorded from E14-15 lumbar ventral roots (Nakayama et al., 1999).

2.7 Survival of transplanted cells does not guarantee axon growth

Nerve transection is used in this study to induce muscle denervation and to mimic the muscle consequences of spinal motoneuron death. After nerve section, the distal stump undergoes Wallerian degeneration, which consists of axon and myelin degeneration (Coleman, 2005; Glass et al., 2002; Mack et al., 2001), macrophage recruitment to cleanup axon and myelin debris by phagocytosis (Mueller et al., 2003; Perry and Brown, 1992), and Schwann cell activation to produce neurotrophic factors and cell adhesion
molecules that promote and guide axon regeneration (Funakoshi et al., 1993, Kirsch et al., 2009). Encouragement of axon regeneration in this peripheral nerve environment diminishes following prolonged periods of axotomy (Fu and Gordon, 1995), in part because Schwann cell numbers decrease (Jonsson et al., 2013). In our model, cells were transplanted into the nerve after one week of degeneration, because a previous study showed that delayed cell transplantation increased the number of axons with large diameters (>6µm; Grumbles et al. 2002). The proximity of the transplant to the denervated muscles also reduced the time available for nerve deterioration.

After injury, peripheral axons start to regenerate at different times. Rate of axon growth also differs (3-4 mm/day, on average; Al-Majed et al., 2000b; Forman and Berenberg, 1978). Growth of axons is supported by cytoskeleton proteins (McQuarrie, 1985), intrinsic factors in the cell body, and the permissive environment in the distal nerve stump, including Schwann cells (Bunge et al., 1986). For example, upregulation of BDNF by Schwann cells promotes axon regeneration and myelination (Bunge, 1987; Cosgaya et al., 2002; Funakoshi et al., 1993).

Axons growth requires additional stimulants even after neuron survival. Axon growth is prompted both by neurotrophic support and activity (Goldberg et al., 2002). Activity leads to elevation of cAMP and to phosphorylation of cAMP response element binding protein (CREB) and c-Jun, two critical transcription factors for the induction of axon regeneration genes (Aglah et al., 2008; Gao et al., 2004; Lyons and West 2011; Raivich et al., 2004). Another target of CREB is BDNF, a neurotrophic factor important for motoneuron survival and neurite growth (Lyons and West 2011). Electrical stimulation of neurons in vivo for at least 30 minutes can sustain the maximum level of
phosphorylated CREB (Fields et al., 1997), and prolongs transcription of BDNF (Bito et al., 1996).

Electrical stimulation of axons in the proximal stump of a cut peripheral nerve for one hour at 20 Hz or 1 Hz increases the number of motor axons that regenerate across the suture line and speeds the return of muscle function (Al-Majed et al., 2000a; Pockett and Gavin, 1985). This effect is mediated by increasing BDNF expression in the cell body and in axons (Eberhardt et al., 2006; English et al., 2007). Various stimulation protocols were used in this study to determine the differential effects of stimulation frequency, pattern, number of pulses, and duration of stimulation on multiple outcomes.

Table 2.1 summarizes the stimulation parameters used in other studies to enhance axon regeneration, improve the return of muscle function, and/or to understand the relationship between various stimulation parameters and the up-regulation of gene expression in neurons (Al-Majed et al., 2000a; Bito et al., 1996; Fields et al., 1997; Pockett and Gavin, 1985). These studies show that better outcomes can be achieved using: 1) different frequencies of stimulation (from 1 Hz to 50 Hz) and number of pulses (from 900 to 1,008,336); 2) a longer duration of electrical stimulation, but within limits (3 min versus 18 s, Bito et al., 1996; >15 min, Pockett and Gavin, 1985; 30 min, Fields et al., 1997; 60 min versus 2 weeks, Al-Majed et al., 2000a); and 3) a shorter rest interval between pulse trains (Bito et al., 1996; Fields et al., 1997). Stimulation frequencies of 1 Hz and 20 Hz for 1 hour were chosen for the current study because these frequencies produce submaximal forces in rat muscles (Fig. 4.4 A and B) and embryonic motoneurons are active spontaneously at low frequencies (Nakayama et al., 1999). Three minutes of stimulation was used in one control group because it is the shortest duration of
stimulation that changed gene expression (Bito et al., 1996) and it controlled for the number of pulses delivered at 1 Hz stimulation for 1 hour. In addition, 1 hour of stimulation was chosen because it produced similar effects to 2 weeks of stimulation (Al-Majed et al., 2000a). A short intervention is also more feasible for clinical application.

2.8 Transplanted motoneurons must retain the ability to innervate muscle to improve muscle function

During development, motoneurons arise from the ventral neural tube under the induction of sonic hedgehog, which is released from the nearby notochord (Arber et al., 1999; deLapeyrière and Henderson, 1997; Tanabe and Jessell, 1996). A gradient of sonic hedgehog expression determines the levels of Pax6, Nkx2.2, MNR2/HB9 and other transcription factors, which direct the ventral neural tube progenitors to become postmitotic motoneurons (Pfaff and Kintner, 1998). In the early phase of motoneuron differentiation, a transcription factor of the LIM homeodomain called Islet-1 is expressed in all motoneurons and is required for their survival (Pfaff et al., 1996). Differential expression of LIM homeodomain proteins (Islet-1, Islet-2, Lim-1, Lim-3) and other transcription factors (e.g. twisted winged helix) determine motoneuron subtypes (Dou et al., 1997; Lumsden, 1995). These gradients of transcription factors and neurotrophic factors change at different stages of development and at various locations, and are important for guiding motor axons to grow along specific paths to reach muscle targets (Bonanomi and Pfaff, 2010). Thus, motoneuron differentiation is a multiple step process that requires precision in both time and location.
### Table 2.1 Stimulation parameters

<table>
<thead>
<tr>
<th>Reference</th>
<th>Frequency (Hz)</th>
<th>Duration</th>
<th>Pulses (n)</th>
<th>Rest between pulses (ms)</th>
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<tr>
<td>Pockett and Gavin, 1985</td>
<td>1</td>
<td>5 min to 60 min</td>
<td>900</td>
<td>1,000</td>
</tr>
<tr>
<td>Fields et al., 1997</td>
<td>10</td>
<td>30 min</td>
<td>18,000</td>
<td>100</td>
</tr>
<tr>
<td>Al-Majed et al., 2000a</td>
<td>20</td>
<td>1 h to 2 weeks</td>
<td>72,000</td>
<td>50</td>
</tr>
<tr>
<td>Bito et al., 1996</td>
<td>5 or 50</td>
<td>18 s or 180 s</td>
<td>900 or 9,000</td>
<td>200 or 20</td>
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</table>
Neuronal transplantation into the spinal cord has been held back by poor motoneuron survival and unclear demonstrations of recovery of muscle function (Blesch and Tuszynski, 2009; Fawcett, 2009). Undifferentiated embryonic stem cells and partially committed neural progenitor cells show poor survival and often uncontrolled differentiation after transplantation. They are more likely to become glial cells, with few retaining a neural phenotype (Bonner et al., 2010; Cao et al., 2001; Parr et al., 2007, 2008; Pfeifer et al., 2004; Vroemen et al., 2007). Cell transplant benefits on host and transplanted neuron survival and tissue repair are related mainly to secretion of growth factors into the cord (Ankeny et al., 2004; Hefferan et al., 2012). Stem cell induced motoneurons have worked when transplanted into developing chick embryos, where many developmental signals and directional limb guidance cues are active and growth distances are short (Amoroso et al., 2013). E14 progenitors suspended in a fibrin gel with growth factors and transplanted into an adult transected spinal cord produced axons that grew long distances and formed some relay circuits, but no reinnervation of muscle was observed (Lu et al., 2012). Functional recovery may also be confounded by sprouting of spared motor axons from the host motoneurons (Deshpande et al., 2006; Kwon et al., 2010; Murray, 2004). It is difficult to distinguish between host axon and axons from transplanted cells unless the transplanted cells are pre-labeled.

Successful efforts to reinnervate muscle have relied on transplantation of embryonic neurons in peripheral nerve, with the transplants composed mostly of ventral glial and neural progenitor cells, with few motoneurons present (Erb et al., 1993; Thomas et al., 2000). Progenitors are poorly committed without the constant presence of such factors as sonic hedgehog (Agalliu and Schieren, 2009; Amoroso et al., 2013; Francius
and Clotman, 2013). For this reason, I explored the potential of using purified motoneurons that are competent to innervate muscle as a primary transplant source (Grumbles et al., 2012). The fate of cells after transplantation is important, as it affects whether or not the cell can perform its intended function in a new environment.

2.9 Neuromuscular junction formation is activity-dependent

Following denervation, acetylcholine receptors are expressed along the muscle fiber membrane (Tsay and Schmidt, 1989), although expression can be clustered by direct electrical stimulation of the muscle (Goldman et al., 1988; Lømo and Westgaard, 1975). Reclustering of acetylcholine receptors is one step in the process of functional muscle reinnervation. Various factors are needed for the clustering of acetylcholine receptors during development and reinnervation, including agrin, muscle-specific kinase (MuSK) and neuregulin. First, the Z isoform of agrin is synthesized by motoneurons and incorporated into the basal lamina of the synaptic cleft (Cohen et al., 1997; Gautam et al., 1996; Reist et al., 1992). Agrin then binds to the lrp4 receptor, and forms a complex with MuSK (DeChiara et al., 1996; Kim et al., 2008). Agrin also regulates neuregulin (Jones et al., 1996; Meier et al., 1997). In addition, colocalization of rapsyn with acetylcholine receptors is required, since rapsyn knockout mice have no acetylcholine clustering at embryonic day 14 even though clustering was observed in control littermates (Gautam, et al., 1995).

Regenerating axons are guided to denervated synaptic sites by Schwann cells that express trophic factors and adhesion molecules (Ramón y Cajal, 1928; Frank et al., 1975; Son and Thompson, 1995). Muscle activity plays an integral role in both the formation
and maturation of neuromuscular junctions, with exchange of signals between the nerve
terminal, the Schwann cell, and the muscle fiber (Nick and Ribera, 2000; Thompson and
Jansen, 1977). These interactions result in the differentiation of both the pre-synaptic
terminal (e.g. accumulation of synaptic vesicles) and the post-synaptic muscle cell (e.g.
formation of junctional folds and accumulation of sodium channels; Cao et al. 2007;
Kelly and Zacks, 1969; Sanes and Lichtman, 1999). Problems with any step in this
process, including an absence of activity, may result in non-functional neuromuscular
junctions, even if a regenerating axon reaches denervated muscle. In our model, the
transplanted neurons do not receive supraspinal and afferent inputs, one factor that may
limit muscle reinnervation and function.

2.10 Denervated muscle undergoes many morphological and physiological changes
Muscle atrophy begins immediately after denervation due to lack of trophic support and
activity. The atrophy is rapid in the first days and weeks, and then the decline slows. If
denervation persists long enough, muscle degenerates (Gutmann, 1948; Gutmann and
The atrophy reduces both twitch and tetanic forces, resulting in higher twitch-tetanic
force ratios (Finol et al., 1981; Hennig and Lømo, 1987; Kotsias and Muchnik, 1987).

Ultrastructural changes include myofibril disruption, sacromere disorganization,
and reduction in mitochondria size (Gauthier and Dunn, 1973; Heck and Davis, 1988; Lu
et al., 1997). Changes in sacroplasmic reticulum cause asymmetrical charge movement
from the transverse tubules to the sacroplasmic reticulum cisternae, which increases
intracellular calcium concentration and slows excitation-contraction coupling, contraction
time and half-relaxation time (Dulhunty and Gage, 1985; Gundersen, 1985; Finol et al., 1981; Salvatori et al., 1988). The global increase in AChR expression throughout the muscle fiber membrane (Tsay and Schmidt, 1989) and the supersensitivity of the muscle to acetylcholine, also contribute to the slow contractions (Rosenblueth and Luco, 1937). Fatigability of muscle increases, in part due to dysfunctional mitochondria, failure of excitation-contraction coupling and fiber weakness (Agbenyega and Wareham, 1990; Ashley et al., 2007a, b; Gundersen, 1985).

The number of satellite cells increases in the first two months after denervation, then steadily declines to below control levels (Mauro, 1961; Schmalbruch and Lewis, 2000; Viguie et al., 1997). The ability of satellite cells to regenerate new myonuclei and muscle fibers may partially explain why muscle fiber size and maximum tetanic force is almost completely restored when muscle reinnervation occurs in less than 1-2 months (Carlson et al., 1996; Gulati, 1990; Gutmann and Zelená, 1962; Kobayashi et al., 1997).

Another obvious functional consequence of muscle denervation is the loss of voluntary muscle control. The stimulus intensity required to excite a muscle rises after denervation, measured by an increase in both rheobase and chronaxie (Albuquerque and Thesleff, 1968; Ashley et al., 2005). Fibrillation potentials start a few days after denervation, and then decrease (Robinson et al., 1991). Decrease in the resting membrane potential and increase in membrane resistance lower excitability by making the membrane more difficult to depolarize (Nicholls, 1956; Ware et al., 1954). This lowered excitability of denervated muscle, coupled with its weakness and fatigability, show why denervated muscle is a poor target for any functional electrical stimulation application.
2.11 Direct electrical stimulation of denervated muscle can reverse some of the
deterioration in muscle structure and properties

Studies have stimulated denervated muscle directly to reduce atrophy and to maintain
contractile properties. As in innervated muscles, effects depend on the stimulation
protocol, the amount of stimulation, and how soon after denervation the intervention is
started (Kernell et al., 1987a, b). Direct muscle stimulation of soleus and extensor
digitorum longus (EDL) muscles in rats and rabbits at high frequency (100 Hz) increased
contractile speed and maximal force but lowered twitch-tetanic ratios, while stimulation
at a low frequency (10 Hz) had the opposite effects (Salmons and Vrbová, 1969;
Westgaard and Lømo, 1988). Stimulation of denervated rat EDL muscles for 5 weeks
maintained cross-sectional area, if more than 50 pulses were delivered each day.
However, maintenance of muscle force required delivery of 200-800 pulses each day
(Dow et al., 2004), suggesting factors other than muscle fiber size are needed to restore
strong muscle contractions. An increase in the amount of stimulation (e.g. number of
pulses) also slows twitches, lowers twitch-tetanic ratios and increases fatigue resistance
(Salmons et al., 1996; Westgaard and Lømo, 1988).

The best time for direct muscle stimulation is immediately after denervation
(Bowden and Gutmann, 1944; Hennig and Lømo, 1987), but electrical stimulation can
also benefit chronically denervated muscles. Direct muscle stimulation after a delay
requires high intensity pulses due to the high excitation threshold of muscle compared to
nerve (Mortimer, 1981), and because muscle excitability is reduced after denervation
(Nicholls, 1956; Ware et al., 1954). Stimulation started 1 month after denervation for up
to 6 months restored rabbit muscle fiber diameter to 72-86% of uninjured values
Stimulation started 2 months after denervation for 5 months increased evoked force in rat soleus and EDL muscles (Hennig and Lømo, 1987). However, when muscles were denervated for much longer times (4-10 months) and then stimulated for up to 2 months, soleus and EDL muscle fiber cross-sectional area, force and specific tension only reached 4-5% of uninjured muscles (Al-Amood et al., 1991; Schmalbruch et al., 1991).

In people with spinal cord injury, Mödlin et al. (2005) showed direct stimulation of denervated quadriceps femoris muscles in both legs for one year increased the cross-sectional area of the muscle in the vicinity of the stimulation electrodes. Since muscle excitability increased over time, 120 ms pulses at 2 Hz for 5 seconds (160 V) were shortened to 40 ms pulses at 20 Hz for 2 seconds. Only individuals with impaired sensation are likely to tolerate this stimulation intervention. Dedication is needed to endure regular daily stimulation. Further, stimulation of muscle denervated longer than 5 years is unlikely to increase muscle fiber cross-sectional area and force due to advanced muscle degeneration and replacement by connective tissue and fat (Kern et al., 2002). All of these limitations imposed by stimulation of denervated muscles have led us to explore repair by muscle reinnervation.

2.12 Early muscle reinnervation is optimal

Denervation is an exclusion criterion for restoration of muscle function by patterned electrical stimulation (Peckham and Knutson, 2005). Muscle reinnervation is important so contractions can be elicited by nerve stimulation, and nerve-muscle trophic interactions can restore many muscle properties. The most visible morphological change
is reduced muscle atrophy, which is accompanied by increases in force. The extent of recovery depends on the period of denervation. If nerve repair is early (within 4-8 weeks of injury), muscle mass and force may be restored completely (Finkelstein et al., 1993; Fu and Gordon, 1995; Gordon and Stein, 1982; Guttmann and Young, 1944; Hennig and Lømo, 1987; Irintchev et al., 1990; Kobayashi et al., 1997). Recovery also depends on muscle use, as muscle fiber size and force are only maintained at 40-50% of control values without activity (Pierotti et al., 1991). In this study the cell transplant is disconnected from the central nervous system. Thus, even after reinnervation, muscle atrophy is expected from lack of muscle use. Poor outcomes after delayed reinnervation may also be explained by decreases in the ability of Schwann cells to release pro-regeneration molecules, increased accumulation of anti-regeneration molecules (e.g. transforming growth factor beta, chondroitin sulphate proteoglycans), immature neuromuscular junctions and muscle fiber atrophy (Jonsson et al., 2013). Höke and Brushart (2010) suggested a delay in reinnervation longer than 6 months in humans results in poor recovery of muscle function, because axons need to grow long distances to reach muscle targets even if nerve repair is immediate. I deal with time and distance issues in this study by transplanting cells near the denervated muscle.

2.13 Measurement of muscle atrophy

Muscle atrophy is often measured by wet muscle weight, but reinnervated muscle may contain a high percentage of connective and adipose tissue, which will lead to an underestimate of muscle atrophy (Schmalbruch and Lewis, 2000). I used muscle fiber cross-sectional area as a measure of muscle atrophy in this study. Fiber size was used to
distinguish reinnervated fibers from denervated fibers. In a previous study, large muscle fibers (fiber cross-sectional area >498 μm²) that were reinnervated were depleted of glycogen by repeated transplant stimulation. These fibers were larger than 95% of fibers in completely denervated muscles (Grumbles et al., 2008). The percentage of large muscle fibers was used as an estimate of the extent of muscle reinnervation. This was favored over NCAM, which is expressed in both disused and denervated muscles (Covault and Sanes, 1985).

2.14 Determinants of muscle force after reinnervation

After human cervical spinal cord injury, muscles scored as normal generate about half the force produced by uninjured individuals. Only 9% of muscle force is needed to move a joint against gravity after spinal cord injury (Needham-Shropshire et al., 1997). Therefore even limited force recovery may be clinically significant. Muscle force is determined by the innervation ratio, muscle fiber size and specific tension (force per cross-sectional area; Burke, 1981). In normally innervated muscles, muscle fiber size is the most important determinant of force, followed by innervation ratio and specific force (Bodine et al., 1987; Tötösy de Zepetnek et al., 1992a). After reinnervation, specific tension is unchanged or slightly increased (Bodine et al., 1987; Tötösy de Zepetnek et al., 1992a). This is also the case in our transplant model (Casella et al., 2010). The percentage of reinnervated muscle fibers (an indirect measure of innervation ratio) is the most important factor in determining muscle force after reinnervation. Denervated muscles are so unexcitable that they will not contribute to muscle force at tolerable stimulation intensities (Mortimer, 1981). One of our goals in stimulating the transplanted cells
electrically was to increase motoneuron survival, which would increase the number of possible connections with reinnervated muscles. Formation of more motor units is critical for recovery of muscle force. Innervation ratios vary from 60-259 in normally innervated rat hind limb muscles, and are highest for large motoneurons (Bodine et al. 1987; Tötösy de Zepetnek et al., 1992a). Innervation ratios can increase within limits, however. Partial denervation studies show that axons can sprout within muscle to innervate up to 5 times more fibers (Brown et al., 1981; Gordon et al., 1993; Thompson and Jansen, 1977). Thus, ~20% of the usual number of motoneurons would have to reinnervate our muscles to restore muscle innervation. In previous studies using the same cell transplant model, it was estimated that approximately 17 motor units can reinnervate all of the muscle fibers in the medial gastrocnemius muscle rather than the usual 82±1 motoneurons (Casella et al., 2010; Grumbles et al., 2012). Reinnervation by fewer motor units has functional consequences. It reduces fine control of muscles because fewer force steps are possible and because activation of each motor unit produces a greater proportion of the whole muscle force.

2.15 Reinnervation may involve muscle fiber type grouping and polyinnervation

Muscle reinnervation commonly involves “type grouping”, or clustering of muscle fibers with a similar metabolic type (Karpati and Engel, 1968; Kugelberg et al., 1970). Single motor unit studies have used glycogen depletion to show that muscle fibers that belong to the same motor units lie close to each other in reinnervated muscles (Bodine et al., 1988; Rafuse and Gordon, 1996; Tötösy de Zepetnek et al., 1992b). These local responses reflect Schwann cells bridges that connect reinnervated to denervated synaptic sites.
(Love and Thompson, 1999). This reorganization also alters motor unit territory from a mosaic to a clustered pattern (Burke, 1981).

One motor end plate can be innervated by multiple axons during development and reinnervation (Brown et al., 1976; Grumbles et al., 2007; Redfern, 1970). This polyneuronal innervation is a sign of an immature neuromuscular junction. Competition between these axons occurs such that only one axon innervates each end plate in adult mammalian muscles and after reinnervation (Brown and Matthews, 1960; Frank et al., 1975; Ijkema-Paassen et al., 2001; McArdle, 1975). For example, polyneuronal innervation averaged 16% in lateral gastrocnemius muscles 7 weeks after transection and self-reinnervation but fell to 3% after 21 weeks (Ijkema-Paassen et al., 2001). I transplanted embryonic ventral spinal cord cells into a peripheral nerve devoid of axons. Although our reinnervated muscle fibers do show signs of polyneuronal innervation, the evoked EMG is consistent in amplitude, suggesting stable muscle activation (Grumbles et al., 2007). In the long-term poly-innervation may be eliminated. Regenerating axons are not forced to innervate the same muscle fibers in our model because some fibers usually remain denervated (Thomas et al., 2000).

2.16 Motoneurons strongly influence the contractile properties of the muscle fibers they innervate

Cross-reinnervation studies show that the force, fatigability and contractile speed of reinnervated muscles depend on the contractile properties of the parent motoneurons (Buller et al., 1960; Chan et al., 1982; Close, 1965; Hennig and Lømo, 1987; Robbins et al., 1969). For example, an extensor digitorum longus (EDL) muscle innervated by soleus
motoneurons will contract more slowly than what is usual for EDL but not as slowly as soleus, and vice versa. The lack of complete conversion is because muscle fibers also impose innate properties on the system.

Other factors also affect muscle contractile properties. Unused muscles with no weight bearing or loading become slower, weaker and more fatigable (Davis and Montgomery, 1977; Pierotti et al., 1991; Spector, 1985). Speed of calcium release and uptake at the sarcoplasmic reticulum is an important determinant of the contraction-coupling mechanism and muscle contractile speed (Rossi and Dirksen, 2006; Stein et al., 1982; Tupling, 2009). This differs for different motor unit types (Stephenson and Forrest, 1980), so it may be important in our model if certain kinds of motoneurons reinnervate the muscles. Reinnervated muscle also becomes more compliant, which will slow contractions (Herbison et al., 1980; Huyghues-Despointes et al., 2003a, b).

Fatigue of muscle depends on many factors, including the task (Bigland-Ritchie et al., 1995). When contractions of paralyzed muscles are evoked by patterned electrical stimulation, force declines are affected by the integrity of the neuromuscular transmission, failure of excitation-contraction coupling, the intrinsic weakness and fatigability of the muscle fibers, blood flow and the oxygen supply (Grumbles et al., 2007; Klein et al., 2006; Thomas and Zijdewind, 2006). Fatigue is also increased at higher stimulation frequencies (Ihlemann et al., 2000; Jones, 1996). Fatigue resistance was measured in this study to determine whether brief transplant stimulation changed the ability of reinnervated muscles to produce force over time.
2.17 Force gradation influences muscle control

The fusion of force in response to higher stimulation frequencies changes force gradation. Fusion is affected by the speed of muscle contraction and relaxation (Chan et al., 1982). For example, a slower motor unit (type S) generates higher relative forces at certain stimulation frequencies compared to a faster motor unit (type F; Botterman et al., 1985; Kernell et al., 1983). The twitch force generated with a single stimulus also affects force gradation. Twitches that produce a higher force, relative to maximum, dramatically reduce the force increases that can be achieved by changing stimulation frequency. Force increases or decreases will be greatest when units operate in the steep part of their force-frequency relationship (Thomas et al., 1991). The number of motor units in a muscle also changes the number of possible force increments in response to stimulation. More motor units in reinnervated muscles will allow finer gradation of force. Since force gradation is important when designing functional electrical stimulation protocols, I counted the number of motor units in reinnervated muscles physiologically.

2.18 Physiological motor unit number estimates

Motoneuron survival is important following transplantation, but from a functional perspective, it is critical to establish the number of transplanted motoneurons that reinnervate a muscle. Motor unit number estimation (MUNE) was first introduced by McComas et al. (1971) to observe the progression of motoneuron death in neuromuscular diseases and aging. Stimulation was delivered to the deep peroneal nerve and its intensity increased in small steps to obtain the first 11 increments in EMG. The MUNE was
obtained by dividing the whole muscle EMG amplitude by the average EMG amplitude for the 11 steps.

Three main assumptions were made: 1) Each increment represents the excitation of another motor unit. This assumption may not be met as motor units can have similar thresholds for excitation. If two motor units have a similar activation threshold, and either unit or both units are activated, 3 increments will result from 2 motor units. The number of possible increments is $2^n - 1$, where $n$ is the number of actual motor units. This “alternation” increases the chance of overestimating the number of motor units when motor unit numbers are higher. 2) The sum of all motor unit EMG potentials recorded separately equals the EMG amplitude elicited from the whole muscle by supramaximal nerve stimulation. If the recording electrodes are placed between the end plates of two motor units, the positive and negative phases of the two EMG recordings could cancel or result in complicated EMG signals. I avoid signal cancellation by using force to estimate MUNE, as in earlier studies (Major et al., 2007; Yang et al., 1990; Thomas et al., 2002). EMG shape and size are secondary criteria. Force steps are also monitored at a common tendon to provide a measure of motor unit size whereas EMG may not be detected if the recording electrodes are placed far away from the active muscle fibers. 3) The EMG amplitudes of the sampled motor units are representative of the total population. Larger nerve fibers have lower thresholds when stimulated electrically (Erlanger and Gasser, 1937). However, proximity of the stimulation electrode to a nerve fiber also changes motor unit activation order in vivo (Thomas et al., 2002), which may bias results. In addition, there are more weak motor units than strong motor units in most muscles (Doherty and Brown, 1997; Thomas et al., 1990), so any sampling method needs to
consider the distribution of motor unit forces within a muscle. For each muscle, I stimulate from subthreshold to supramaximal so all motor units can be sampled (Thomas et al., 2002). Thus, our methods address signal cancellation and distortion, as well as sampling bias, but are susceptible to alternation. I use three series of pulses with different pulses durations (10, 20, 50 µs) so a similar force increment across the pulse series likely represents activation of the same motor unit or groups of units. Further, activation thresholds are separated more with shorter pulse durations (Mortimer, 1981).

2.19 Alternative MUNE methods to minimize alternation

To minimize alternation, Brown and Milner-Brown (1976) averaged single motor unit EMG potentials in response to low intensity stimulation at different positions along the nerve (the multiple point stimulation method). Total EMG amplitude was divided by the number of increments. This method fails to address the magnitude of alternation. Further, our cell transplants are only 2-5 mm, so it is difficult to selectively stimulate the transplant at distinct sites.

Slawnych et al. (1996) attempted to minimize alternation by assuming that all EMG increments measured at constant stimulation intensity could be decomposed into individual motor unit potentials. Up to 3 motor units with overlapping activation thresholds could be recognized and 10-15% of possible responses were sampled. Issues of signal cancellation may also affect the decomposition process.

Stashuk et al. (1994) used the mean F-wave amplitude to estimate the number of motor units. When motor axons are electrically stimulated, orthodromic action potentials excite the muscle to produce a contraction, and antidromic action potentials go to the
motoneuron cell bodies in the spinal cord. If a motoneuron is re-excited, a second
orthodromic action potential will activate the muscle fibers again and produce an F-wave.
Since only a portion of motoneurons produce F-waves at a given time, sampling bias is
an issue. There is no guarantee that each F-wave or increment represents a single motor
unit, nor does this method address potential signal cancellation.

2.20 Statistical MUNE methods
Statistical methods have assumed the increments in EMG follow a Poisson distribution
(Daube, 1995) or a binomial distribution (Blok et al., 2005). These methods sampled
motor units over a wider range of stimulation intensities and were more objective,
because they did not require the experimenter to determine the response of a single motor
unit. However, each motor unit is assumed to be of similar size, which is rarely the case
(Doherty and Brown, 1997; Thomas et al., 1990).

A more recent statistical method utilized a Bayesian approach to estimate the
most probable number of motor units in a muscle (Ridall et al., 2006). This approach
incorporated baseline noise to account for some of the response variance for a given
motor unit, used the whole range of stimulation intensities to address sampling bias and
variability in motor unit size, and considered motor units with overlapping excitation
curves to account for alternation. The issue of signal cancellation with EMG signals
remained. EMG signals are also influenced by volume conduction of signals from the
active fiber to the muscle surface and so are a poor estimate of motor unit size (Bigland-
Ritchie et al., 1981). If this Bayesian approach could utilize force responses, it would
address all the issues of variability in size within and across motor units, alternation, signal cancellation, signal distortion from volume conduction, and sampling bias.
Electrical Stimulation of Embryonic Neurons for One Hour Improves Axon Regeneration and the Number of Reinnervated Muscles that Function

Background

Motoneuron death occurs following trauma to the spinal cord and in neurological diseases such as amyotrophic lateral sclerosis and poliomyelitis (Bradley et al., 1983; Sharrard, 1955; Thomas and Zijdewind, 2006). Muscles are denervated and paralyzed, eliminating voluntary movements (Mulcahey et al., 1999; Gutmann, 1948). Muscle atrophy begins immediately and eventually results in muscle degeneration if no reinnervation occurs (Degens et al., 2008; Finkelstein et al., 1993). Since adult spinal motoneurons do not regenerate, motoneuron replacement becomes an important option to promote functional recovery (Rowland et al., 2008).

While many studies of spinal cord injury (SCI) focus on central nervous system axon regeneration to restore connections between brain and muscle, surveys of people with SCI suggest research should also be focused on functions that would improve their quality of life now, including arm, hand, bladder, bowel and sexual function, and neuropathic pain relief (Anderson, 2004; Estores, 2003; Widerström-Noga et al., 1999). Use of patterned electrical stimulation to activate muscles locally, termed functional electrical stimulation (FES), has produced many success stories, particularly in the restoration of upper and lower extremity function, bladder, and bowel function and

respiratory function (Peckham and Knutson, 2005). However, FES applications require innervated muscles. Muscle denervation is an exclusion criterion for FES because the excitation threshold for muscle is substantially higher than that for nerve. With the high currents needed to excite denervated muscles, it is difficult to achieve selective muscle activation or strong contractions (Mortimer, 1981). Since complete muscle denervation occurs in 1 out of 6 people with SCI, these individuals could not be helped by FES unless the nerve supply was restored to the target muscles (Thomas and Zijdewind, 2006).

In animal models, various cells have been transplanted to replace motoneurons (e.g. neural progenitor cells, bone marrow stromal cells, embryonic stem cells) and to enhance axon regeneration (e.g. Schwann cells, olfactory ensheathing cells; Abematsu et al., 2010; Deshpande et al., 2006; Li et al., 1997; Lu et al., 2012; Ramon-Cueto et al., 1998; Reier, 2004; Sandner et al., 2012; Thomas and Moon, 2011; Wyatt et al., 2011). In these models, the injury severity is often mild to moderate. Secondary effects such as inflammation may not be as severe as the spinal contusion that is typical of human SCI (Bunge et al., 1993; Siegenthaler et al., 2007). Mild and moderate injury also leaves spared motoneurons and axons, making it difficult to interpret the effectiveness of treatments (Kwon et al., 2010; Murray, 2004). For example, motor axons that survive the injury can sprout within partially denervated muscle to reinnervate up to 5 times their usual number of muscle fibers (Thompson and Jansen, 1977), but this natural recovery process does not arise from any treatment. In addition, motor axons are usually required to grow over long distances to reinnervate muscle, and a prolonged period of denervation results in increasingly severe muscle atrophy (Gutmann and Young, 1944, Thomas et al.,
Without significant restoration of muscle mass, reinnervation will produce little effect on muscle function.

To minimize the amount of muscle atrophy after complete denervation, a local motoneuron replacement strategy was previously developed to reinnervate skeletal muscles as soon as possible. Ventral spinal cord cells isolated from rat embryos were transplanted into the tibial nerve near the denervated muscles (Erb et al., 1993; Thomas et al., 2000). Muscle atrophy was reduced following reinnervation, but poor survival of transplanted motoneurons limited the number of reinnervated muscle fibers (Grumbles et al., 2002). Motoneuron survival, axon regeneration, muscle reinnervation and function were all improved when three neurotrophic factors were added to the cells (glial-derived neurotrophic factor, GDNF; hepatocyte growth factor, HGF; insulin-like growth factor-1; IGF-1; Casella et al., 2010; Grumbles et al., 2009). Activity is also crucial for the survival of embryonic motoneurons (Brunet et al., 2007; Gallo et al., 1987; Goldberg et al., 2002; Perez-Garcia et al., 2008) and may promote activity-dependent axon growth (Ben-Ari, 2001). Al-Majed et al. (2000a) found that brief stimulation of the proximal stump of the transected femoral nerve at 20 Hz for 1 hour increased the number of motoneurons that initiated axon regeneration across the suture line. Pockett and Gavin (1985) found that stimulation of the sciatic nerve at 1 Hz for 1 hour proximal to a nerve crush sped up the return of motor function. Nakayama et al. (1999) reported that rat lumbar ventral roots were spontaneously active at embryonic day 14.5. Thus, treatments that activate multiple pathways through both membrane depolarization and neurotrophic factors may enhance neuron survival and axon regeneration further (Brunet et al., 2007; Kanzaki et al., 2002).
The aim of this study was to determine whether brief electrical stimulation of transplanted ventral spinal cord cells immediately after they were put into the tibial nerve increased motoneuron survival, axon regeneration, muscle reinnervation and function 10 weeks later. Various protocols were used to examine the differential effects of stimulation frequency, pattern, number of pulses, and duration of stimulation on the motoneuron count, myelinated axon count, muscle fiber area, the extent of reinnervation, and the percentage of muscles that contracted in response to stimulation of the neuron transplant.

Materials and methods

Animals

All procedures performed on animals were approved by the University of Miami Animal Care and Use Committee and adhered to the animal care and use guidelines of the National Institutes of Health. Inbred female Fischer rats (3 months old, mean±SE weight: 163±2 g) and day 14-15 embryos from pregnant rats (Harlan Laboratories, Indianapolis, Indiana) were used in this study to prevent immune rejection of the transplanted cells.

Experimental groups and timeline

This study is based on a cell transplant strategy that involves injection of dissociated embryonic ventral spinal cord cells into the tibial nerve of adult rats as the only source of neurons to reinnervate hind limb muscles (Thomas et al., 2000). Animals underwent denervation (week -1), transplantation and intervention (week 0), and physiological assessments followed by tissue retrieval for analysis (week 10; Fig. 3.1). Eight groups of
animals were studied (Table 4.1). The electrical stimulation intervention was applied immediately after cell transplantation and involved stimulation at: 1) 20 Hz for 1 hour (High frequency group) because more axons started to grow across the suture line when this stimulation was applied to the proximal stump of the transected femoral nerve (Al-Majed et al., 2000a); 2) 1 Hz for 1 hour (Low frequency group) because this stimulation sped up the return of motor function when delivered to the sciatic nerve proximal to a nerve crush (Pockett and Gavin, 1985); 3) 20 Hz for 6 seconds, once every 2 minutes, for 1 hour (Intermittent group) to mimic the spontaneous activity of motoneurons at embryonic day 14-15 (Nakayama et al., 1999); 4) Scrambled frequencies (Scrambled group), which varied between 11 and 30 Hz, with a mean frequency of 20 Hz for 1 hour, were generated using a random number generator (random.org). This group controlled for stimulation pattern and frequency (20 Hz) because the protocol included the same total number of pulses (n=72,000) as was delivered in the High frequency group but stimulation pattern differed; 5) 20 Hz for 3 minutes (Duration control) which was designed to distinguish the effects of stimulation duration while keeping the frequency the same as the High frequency group and the total number of pulses (n=3,600) the same as the Low frequency and Intermittent groups; 6) Placement of the tibial nerve on the electrodes for 1 hour but no stimulation was delivered to control for the stimulation (No stimulation group); 7) Injection of media (No cells) to control for the effects of cell transplantation. The nerve was also placed on the electrodes for 1 hour without delivery of stimulation; and 8) Naïve, age-matched animals that received no cell transplantation or electrical stimulation (Uninjured Group). These animals were used for tissue and physiology assessments only.
Table 3.1 Experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Denervate</th>
<th>Transplant</th>
<th>Frequency (Hz)</th>
<th>Time (min)</th>
<th>Pulses (n)</th>
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<td>+</td>
<td>20</td>
<td>60</td>
<td>72,000</td>
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<tr>
<td>Low frequency</td>
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<td>+</td>
<td>1</td>
<td>60</td>
<td>3,600</td>
</tr>
<tr>
<td>Intermittent</td>
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<td>+</td>
<td>20 Hz for 6 s, once/2 min</td>
<td>60</td>
<td>3,600</td>
</tr>
<tr>
<td>Scrambled</td>
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<td>+</td>
<td>Mean = 20</td>
<td>60</td>
<td>72,000</td>
</tr>
<tr>
<td>Duration control</td>
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<td>+</td>
<td>20</td>
<td>3</td>
<td>3,600</td>
</tr>
<tr>
<td>No Stimulation</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>No cells</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Uninjured</td>
<td>—</td>
<td>—</td>
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Figure 3.1 Experimental design. At week -1, the hind limb muscles were denervated by sciatic nerve transection. The proximal stump was tied to hip muscles to prevent reinnervation from spinal motoneurons. One week following denervation, 200,000 embryonic day 14-15 (E14-15) rat ventral spinal cord cells, purified for motoneurons, were injected into the tibial nerve with neurotrophic factors. The cells were either stimulated immediately after transplantation or the electrodes were placed under the tibial nerve but no stimulation was delivered. At week 10, EMG and force were recorded in response to transplant stimulation. The transplant, nerve branches, and muscles were taken for analysis.
Muscle denervation

Animals were anesthetized with sodium pentobaritol (40 ml per kg, IP) during all surgeries. Body temperature was maintained between 35-37 °C with a heating pad. The left sciatic nerve was transected 15-20 mm proximal to where the tibial nerve enters the gastrocnemius muscles. This denervates many hind limb muscles and mimics the muscle consequences of motoneuron death. The proximal nerve stump was tied to the adductor femoris muscle so spinal motoneurons were unable to reinnervate the denervated muscles.

Cell preparation

Ventral spinal cord cells were dissociated from day 14-15 embryos (Camu and Henderson, 1992; Grumbles et al., 2002). The embryos were placed into calcium and magnesium free phosphate buffered saline (PBS) solution. The ventral spinal cord was dissected out and incubated in trypsin solution at 37 ºC for 15 minutes, washed three times in Leibowitz-15 (L15) medium (Invitrogen, Carlsbad, CA), transferred into deoxyribonuclease (DNase) solution, and triturated using a glass pipette in bovine serum albumin (BSA). Motoneurons were purified from these ventral spinal cord cells using a density gradient centrifugation technique (Taylor et al., 2007). The cells were centrifuged at 913g for 15 minutes and the layer of motoneurons between the Optiprep solution and medium was collected. These cells were suspended in 4% BSA containing L15 and centrifuged at 230g for 10 min. The purified motoneuron pellet was then re-suspended in L15. Brain-derived neurotrophic factor (BDNF, 10 ng/ml), ciliary neurotrophic factor (CNTF, 10 ng/ml), GDNF (10 ng/ml), HGF (10 ng/ml), IGF-1 (10 ng/ml), and forskolin
(10 µM) were added to the cell medium since these factors increased spinal motoneuron survival *in vitro* (Hanson et al., 1998) and motoneuron survival in our model (Grumbles et al., 2009). Cells were counted with a hemocytometer and viability estimated using Trypan Blue.

**Cell transplantation and brief electrical stimulation**

Cell transplantation was delayed for one week after denervation because this improved axon regeneration (Grumbles et al., 2002). At week 0, 200,000 ventral spinal cord cells (73±2% of cells were positive for Islet-1, a marker for embryonic motoneurons; Grumbles et al., 2012) were suspended in 5 µl of medium and injected into the tibial nerve 10-15 mm from where it enters the gastrocnemii muscles using a Hamilton syringe. Immediately following transplantation, a pair of silver electrodes was placed under the tibial nerve where cells (or medium) had been transplanted. All stimulation pulses were delivered at 3 V (100 µs pulse duration; Al-Majed et al., 2000a) using a S48 stimulator (Grass Medical Instruments, Quincy, MA). This stimulation intensity is supramaximal (~150%) for excitation of all axons in rat sciatic nerve. Frequency, duration and stimulation pattern were adjusted for each treatment group (Table 3.1).

**Physiological assessments**

Ten weeks following transplantation of cells or medium, reinnervation and function of the medial gastrocnemius (MG), lateral gastrocnemius (LG) and plantaris (PL) muscles of the left leg were assessed physiologically. The tibial nerve and the test muscles were separated by dissection. The left knee and ankle were clamped to prevent movement
during the recordings. Suture (USP 4-0) was tied to the tendon of the each muscle, and in turn, to a force transducer to record isometric force. Both the separation of test muscles, and their independent connection to the transducer, minimized recording force from non-test muscles (contamination) during evoked contractions. A pair of silver electrodes was placed on the mid belly of each muscle to record electromyographic activity (EMG). The separation of muscles minimized volume conduction of electrical signals generated by one muscle to another muscle (signal contamination). The cell transplant was laid on a pair of silver electrodes for stimulation. Muscle temperature was kept between 35-37 °C with a heating lamp and monitored using a metal temperature sensor (Fine Science Tools, Foster City, CA).

First, the optimal muscle length for force generation was found by delivering single pulses to the transplant (30 V, 50 µs duration). Muscle length was changed in 1 mm steps until maximal twitch force was evoked. To examine whether or not MG, LG and PL functioned, pulses (50 µs) were delivered at increasing intensity (in 1 V increments to 30 V, then in 10 V increments to 150 V) while recording EMG and force. A muscle was considered functional if stimulation at 150 V (50 µs pulses) elicited a muscle contraction, judged by the presence of both EMG and force. To determine the maximum evoked MG force, trains of stimuli were delivered at 40 Hz and 50 Hz for 1 s and at 100 or 200 Hz for 0.5 s. To examine whether the stimulation treatment altered the function of neuromuscular junctions, EMG and force were recorded in response to repeated transplant stimulation (13 pulses at 40 Hz, every second for 2 minutes). The fatigability (final force/initial force; or EMG area) of the reinnervated muscles is reported
in detail elsewhere. EMG and force were sampled online using a SC/Zoom system (3,200 and 400 Hz respectively; Umeå University, Sweden).

**Motoneuron survival**

Following the physiological recordings, the transplant was removed, fixed in Zamboni’s solution (paraformaldehyde and picric acid; Newcomer Supply, Middleton, WI) for 2 days, placed in PBS solution containing 10%, 20%, then 30% sucrose (1 day each), frozen in dry-ice cooled isopentane, and stored at -80 °C. Transverse sections (25µm thick) of the entire transplant were cut and placed on gelatin coated slides in such a way that each slide had sections that were representative of the entire transplant. This was achieved by cutting from proximal to distal, by putting one proximal section on all slides first, then returning to the first slide to place a second section and so on. Thus, the second section on the first slide came from a location well within the transplant. Sections were stored at -80 °C.

Slides were washed with PBS, treated in -20 °C methanol for 20 minutes, incubated in borohydrite for 20 minutes to block background fluorescent staining, exposed to sodium dodecyl sulfate (SDS) detergent for 12 minutes, then kept in 1% milk solution overnight to block non-specific protein sites. The slides were immunostained with anti-ChAT primary antibody (choline acetyltransferase; dilution 1:90, Cat #: AB143, Millipore, Billerica, Massachusetts) overnight for 24 hours. On the third day, slides were washed in PBS, stained with 1:120 goat anti-rat Alexa 488 secondary antibodies (Invitrogen) for 4 hours, then mounted using Prolong Gold antifade agent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen).
A systematic random sampling method was used to estimate the number of ChAT-positive neurons in the whole transplant (Oppenheim et al., 1989), a measure of motoneuron survival. Analysis of motoneurons and all other outcome measures described below were completed blind to group. For every 3rd section, images of ChAT-positive neurons with DAPI stained nuclei were digitized at 40x magnification using a Axiphot microscope (Zeiss, Jena, Germany), a MicroFire A/R camera (Optronics, Goleta, CA), and Stereo Investigator software (MBF Bioscience, Williston, VT). The diameter of each ChAT-positive neuron was measured using Metamorph software (Molecular Devices, Sunnyvale, CA). The total number of ChAT-positive neurons in a transplant was estimated by dividing the number of sampled neurons by the fraction of sections sampled. Motoneuron survival may be influenced by astrocytes (Eagleson et al., 1985; Taylor et al., 2007). Since I transplanted cells into the tibial nerve, a Schwann cell dominant environment, representative transplants from the Low frequency and No stimulation groups (n=7) were stained with aldehyde dehydrogenase 1A1 (ALDH1A1, P28307, NeuroMab, Antibodies Inc, Davis, CA). This antibody was used to detect the presence of astrocytes in the cell transplant, but is negative for oligodendrocytes, Schwann cells and neurons (Adam et al., 2012; Cahoy et al., 2008). These same transplants were also stained for glial fibrillary acidic protein (GFAP; rabbit anti-glial fibrillary acidic protein, Z0334, DAKO Corporation, Carpinteria, CA), an antibody that recognizes Schwann cells, oligodendrocytes and most astrocytes (Eng et al., 2000).
Axon regeneration

Axon regeneration was first analyzed by counting the number of myelinated axons in the MG, LG and distal tibial nerves (DT; the tibial nerve that remains after MG and LG nerves branch to their respective muscles). These three nerve branches were removed following the physiological recordings and submerged in Zamboni’s solution. Two days later, the tissue was washed twice with cold PBS solution and stored in 4% glutaraldehyde. Fixed nerve sections were stained with 1% osmium tetraoxide in 0.1 M PO₄ buffer overnight, dehydrated using a series of alcohols (25%, 50%, 70%, 95%, 100%), washed with propylene oxide, and kept in a 1:1 mixture of propylene oxide and Epon Araldite resin overnight. The nerve sections were then embedded in Epon Araldite resin and incubated at 60 °C overnight to harden.

Nerves were cut transversely (1 µm), stained with Toluidine blue to show myelin, and mounted using DPX mounting medium (Electron Microscopy Science, Hatfield, PA). Sections were digitized (63x magnification), as described for motoneurons. The maximum and minimum diameter of each myelinated axon was measured (Metamorph software) and average diameter was calculated. These data also provided the axon count for each nerve branch and the total number of axons that regenerated from the transplant.

The stimulation treatment may influence axon regeneration, myelination or both processes so representative MG nerves from the Low frequency and No stimulation groups were also examined at the electron microscopic level. Unmyelinated and myelinated axons were counted in up to 23 samples per nerve to assess overall axon regeneration. To examine the amount of myelination, the axon diameter and myelin


thickness of myelinated axons were measured in these same samples. The g ratio (axon diameter/fiber diameter) was calculated as a measure of myelin thickness.

**Muscle atrophy and reinnervation**

Muscle atrophy was determined by measuring the cross sectional area of muscle fibers in MG muscles. Following the physiology, the left MG muscle was dissected free, frozen in dry-ice cooled isopentane, and stored at -80 °C. Cross sections (10 µm) were cut from the mid belly of each muscle and stained with Harris Hemotoxylin (Electron Microscopy Science) for 3 minutes, washed with water for 10 min, stained in Eosin for 1 minute, washed with water for another 3 min, dehydrated in 70%, 90%, 100% reagent alcohol, washed in citrate solution for 5 minutes, then mounted with DPX mounting medium (Electron Microscopy Science).

Muscle cross sections were digitized at 40x magnification. For each muscle, 500 fibers were randomly sampled across the entire section and the area of each muscle fiber measured (Metamorph software). A grid was laid over the muscle cross-section, with each square subdivided into smaller regions. A number between 1 and 3 was selected randomly and the areas of up to 5 muscle fibers were measured in the middle of the selected region. Muscle reinnervation was determined from the percentage of large muscle fibers. We have previously stimulated cell transplants repeatedly to deplete functional (reinnervated) muscle fibers of glycogen. Glycogen-depleted (reinnervated) fibers had muscle fiber areas larger than 95 % of fibers in completely denervated muscles (fiber cross-sectional area >498 µm²; Grumbles et al., 2008).
Statistics

Mean ± standard errors (SE) are given. Statistics were performed using PASW Statistics 18 (SPSS). The mean number of ChAT-positive neurons, myelinated axon counts, muscle fiber area, percentage of reinnervated muscle fibers, and maximum muscle force were compared across groups using one-way ANOVA. Unmyelinated axon counts, g ratios and fatigue indices were compared for only the Low frequency and No stimulation groups. The percentage of functional muscles (MG, LG, PL) was compared across groups using Chi-square tests. Correlations between myelinated axon counts, muscle fiber area, muscle reinnervation and maximal muscle force were compared using Pearson correlation analysis. Level of significance was P<0.05.

Results

Electrical stimulation of transplanted cells at 1 Hz for 1 hour increased the number of myelinated axons at 10 weeks

Myelinated axons were present in the tibial nerves of animals that received a cell transplant whether or not a brief stimulation treatment was delivered (Figs. 3.2A, B). However, these myelinated axons were smaller than axons in nerves of uninjured animals (Fig. 3.2D). When medium was transplanted without cells, the tibial nerve was devoid of myelinated axons, so muscles in these animals remained denervated (Fig. 3.2C). The number of myelinated axons was higher in the Low frequency (1 Hz for 1 hour) group. The mean total number (±SE) of myelinated axons in the tibial nerve was 727±109, which was significantly higher than the axon count for the Duration control (20 Hz for 3 min; 480±67), Intermittent (460±77), and No stimulation groups (415±122; Fig.
Figure 3.2 Axon regeneration. Cross sections (1 µm thick) of MG nerves stained with toluidine blue to show myelinated axons. Sections are from animals that received a transplant of cells with electrical stimulation at 1 Hz for 1 hour (A), cells without electrical stimulation (B), medium but no cells (C), and an uninjured animal (D). (E) Mean (± SE) total count of myelinated axons in the tibial nerve at 10 weeks (*P<0.05, Low frequency count compared to Intermittent, Duration control, and No stimulation groups; **P<0.01, Uninjured count compared to all other groups, one-way ANOVA). Numbers at the bottom of each bar indicate the number of animals per group. There were no myelinated axons in the No cells group. (F and G) Mean myelinated axon counts in different nerve branches across groups (Both P<0.05, Pearson correlation analysis; R²=0.86 in F, R²=0.78 in G). DT, distal tibial nerve. Short group names are used in this and subsequent figures (High, High frequency; Low, Low frequency, Int, Intermittent; Scr, Scrambled; Dur, Duration control; No Stim, No Stimulation).
The mean number of myelinated axons in all experimental groups, including the Low frequency group, was lower than that for the Uninjured group (1160±33), suggesting that further improvements in axon regeneration are possible.

Comparisons of myelinated axon numbers in different nerve branches (MG, LG, DT: i.e. tibial nerve distal to where MG and LG nerves branch to their respective muscles) show significant positive correlations between the number of myelinated axons in each nerve branch (Figs. 3.2F, G). Thus, if the number of myelinated axons was high in one nerve branch for a given animal, the axon count was likely to be high in the other nerve branches of that same animal. Furthermore, the mean MG myelinated axon count for the Low frequency group was 50% more compared to the No stimulation group (77 ± 23 versus 51±19, P<0.05, Fig 3.2F), but the estimated unmyelinated axon counts were similar (309±139 versus 336±133). These results suggest that more axons grew from the transplants and became myelinated with Low frequency stimulation. This same treatment did not alter the myelin thickness of axons, however. Axon diameter and myelin thickness were correlated positively for both the Low frequency and No stimulation groups, and the respective mean g ratios were similar (0.62±0.01 versus 0.59±0.03).

One hour of electrical stimulation increased the percentage of functional muscles
Medial gastrocnemius (MG), lateral gastrocnemius (LG), and plantaris (PL) muscles were classified as functionally reinnervated 10 weeks after transplantation when stimulation of the transplant (150 V, 50 µs) elicited an EMG response and force. Since the proximal sciatic nerve stump was tied to hip muscles and remained in place in all
animals, these evoked contractions did not arise from exciting axons belonging to spinal motoneurons.

Stimulation of the cell transplant for one hour immediately after transplantation enhanced functional muscle reinnervation (Fig. 3.3). The percentage of functionally reinnervated muscles was significantly higher for animals in the High frequency (79%), Low frequency (77%), Intermittent (87%), and Scrambled (86%) groups compared to animals in the Duration control (64%) and No stimulation groups (58%). This result suggests that duration of stimulation (1 hour) was a more important factor for functional muscle reinnervation than the number, frequency, or pattern of pulses because the High frequency, Scrambled and Intermittent groups received more pulses (n=72,000) than the Low frequency group (n=3,600), at a higher frequency (20 Hz versus 1 Hz), and the pulses were delivered using different patterns of stimulation.

**Cell transplantation resulted in muscle reinnervation which reduced atrophy**

Animals that did not receive a cell transplant had small muscle fibers in MG due to long-term denervation (Fig. 3.4A). Fibers in muscles of uninjured animals were large (Fig. 3.4C). Muscles of animals that received a cell transplant had large and small muscle fibers (Fig. 3.4B), an indication that only some muscle fibers were reinnervated (Grumbles et al., 2008). The larger muscle fibers were often grouped together, suggesting that regenerating axons sprouted to innervate neighboring muscle fibers.

The mean (±SE) cross-sectional area of reinnervated muscle fibers was similar across all groups that received a cell transplant, ranging from 36±1 % to 44±4 % of the mean uninjured value (Fig. 3.4D). The mean muscle fiber areas of these cell
**Figure 3.3** Percent of functional muscles. Mean (±SE) percentage of muscles that were reinnervated and contracted in response to transplant stimulation (150 V, 50 µs pulses) at 10 weeks. Numbers at the bottom of each bar indicate the number of animals per group. MG, LG and PL muscles were tested in each animal. (**, P<0.01, Chi-square test).
Figure 3.4 Reinnervation and muscle atrophy. Hematoxylin & eosin stained cross-sections (10 µm) of a denervated (medium injected without cells, A), reinnervated (20 Hz 1 hour, B), and uninjured MG muscle (C). (D) Mean (±SE) area of reinnervated MG muscle fibers (>498 µm² cross-sectional area; 50) and percentage of reinnervated MG muscle fibers (E) across groups compared to denervated fibers (No cells; *, P<0.05, one-way ANOVA). Numbers at the bottom of each bar indicate the number of animals per group.
transplantation groups were significantly larger than those of the group that received medium without cells. Thus, reinnervation from the cell transplantation alone increased muscle fiber areas significantly but brief electrical stimulation of the embryonic cells did not reduce muscle atrophy further.

The mean (±SE) percentage of muscle fibers that were reinnervated was similar across all groups that received cells whether or not stimulation was delivered immediately after transplantation. Reinnervation ranged from 33±4 % to 48±5 % across groups (Fig. 3.4E).

**Greater axon regeneration improved muscle function**

Correlations between parameters were examined for MG muscles. There were significant positive correlations between the mean myelinated axon counts in MG nerves, reinnervated muscle fiber areas, the percentage of reinnervated fibers and mean maximum muscle forces (Fig. 3.5). Muscles in the Low frequency group (1 Hz for 1 hour) generated the most force and had the highest myelinated axon count. There were fewer axons in the MG nerves of the other groups, and the mean maximum evoked muscle force decreased proportionally (Fig. 3.5A). Muscle fiber area and the extent of muscle reinnervation both influenced the maximum MG muscle force strongly and were higher for the High and Low frequency groups, intermediate for the Scrambled, Intermittent and No stimulation groups, and lowest when the transplant was stimulated for 3 minutes (Duration control; Figs. 3.5B, C).
Figure 3.5 Correlations between parameters. Mean maximal MG force across groups as a function of MG myelinated axon count (A), reinnervated muscle fiber area (B), and percentage of reinnervated muscle fibers (C). (P<0.05, Pearson correlation analysis; $R^2=0.86$ in A, $R^2=0.86$ in B, $R^2=0.97$ in C).
**Brief electrical stimulation did not change motoneuron survival**

Motoneuron survival was estimated in transplants at 10 weeks by counting the number of ChAT-positive neurons that had a visible nucleus (Figs. 3.6A-C). The estimated mean (±SE) motoneuron count was similar across groups and ranged from 281±107 in the Scrambled group to 426±184 in the No stimulation group (Fig. 3.6D). There were no motoneurons present in the tibial nerve when only medium was transplanted without cells. These data suggest motoneuron survival was not increased by brief electrical stimulation of the cell transplant. Further, transplants from the Low frequency and No stimulation groups that resulted in functional muscle reinnervation were devoid of astrocytes, except in one case, where a small area was positive for ALDH1A1, a marker for astrocytes. These same transplants were all positive for GFAP which recognizes Schwann cells and most astrocytes (Eng et al., 2000).

**Discussion**

**Brief electrical stimulation of embryonic neurons in peripheral nerve had long term effects**

I have shown that brief electrical stimulation of embryonic ventral spinal cord cells immediately following transplantation into peripheral nerve had long-term effects on axon regeneration, with subsequent improvements in muscle function. More axons grew from the transplants when they were briefly stimulated at a low frequency (1 Hz) for one hour. The duration of stimulation was also important because there were more functional muscles at 10 weeks when the neurons were stimulated for one hour after transplantation.
Figure 3.6 Motoneuron survival. Sections stained with ChAT antibody (A) and DAPI (B) were merged (C) to verify that only ChAT-positive neurons with a clear nucleus were counted to estimate motoneuron survival. Estimated mean (±SE) number of motoneurons in cell transplants across groups. Only transplants of animals with functional MG muscles were assessed. Numbers at the bottom of each bar indicate the number of animals assessed per group.
Electrical stimulation influences multiple pathways

Electrical stimulation induces membrane depolarization and calcium influx but the effects depend on the route of calcium entry. Influx of calcium through N-methyl D-aspartate (NMDA) glutamate receptors can mediate excitotoxic cell death (Choi, 1988; Hartley et al., 1993). However, when calcium influx occurs via L-type voltage-gated calcium channels, this activates adenylate cyclase, induces the production of cyclic adenosine monophosphate (cAMP), increasing both neuron survival and axon regeneration (Gallo et al., 1987; Collins and Lile, 1989; Cooper et al., 1995; D’Mello et al., 1993; Koike et al., 1989; Meyer-Franke et al., 1995; Rydel and Greene, 1988). Calcium can also bind to calmodulin dependent protein kinases (CaMK) to promote neuron survival (Hack et al., 1993; Hanson and Schulman, 1992). Activation of these pathways influences transcription factors such as cAMP-responsive element binding protein (CREB) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to increase the transcription of factors such as BDNF to enhance axon regeneration (Goldberg et al., 2002; Polleux and Snider, 2010; Zheng and Poo, 2007). Both electrical stimulation and neurotrophic factors can activate the Ras/Raf and phosphatidylinositol-3-OH kinase (PI3K) pathways to enhance axon regeneration (Bonni et al., 1992; Dudek et al., 1997; Lev et al., 1995; Miller et al., 1997; Rosen et al., 1994; Xia et al., 1995; Yao et al., 2003). The effects of electrical stimulation on axon regeneration also occur via enhanced sensitivity of neurotrophic receptors (Goldberg et al., 2002). Thus, treatments that activate multiple pathways, through both membrane depolarization and neurotrophic factors may enhance axon regeneration further (Brunet et al., 2007; Kanzaki et al., 2002). Consistent with this idea, all cell transplants included neurotrophic factors in this study.
and there were more axons that grew from transplants that were stimulated at a low frequency for one hour (Fig. 3.2E).

Restoration of intracellular calcium levels via electrical stimulation may also enable neuron survival by helping neurons reach a stable membrane potential. The cell isolation process induces axotomy and ruptures the cell membrane. Prompt membrane resealing is necessary to maintain the intracellular ionic balances needed to conduct action potentials and stimulation-induced calcium release may enhance membrane reconstruction (Bloom and Morgan, 2011; Xie and Barrett, 1991). Elevation of intracellular calcium concentration can also activate the polymerization of microtubules and actin filaments required for axon growth cone initiation and extension (Dehmelt and Halpain, 2004; Gitler and Spira, 1998). Repellent signals for growth cone extension can be changed into attractive signals by electrical stimulation, which may facilitate axon regeneration (Ming et al., 2001). Thus, from the multitude of ways electrical stimulation may work, ultimately, it is maintaining or restoring the level of intracellular calcium and/or cAMP to physiological levels that is likely to be the key factor in facilitation of motoneuron survival or axon regeneration (Kater and Mills, 1991; Moore and Goldberg, 2010; Moulder et al., 2003; Rehder et al., 1992).

Myelinated axon counts were higher with brief transplant stimulation at a low frequency

Growth cones of embryonic motoneurons show faster rates of outgrowth when the frequency of calcium transients is lower (Gomez and Spitzer, 1999). Stimulation (1 Hz) of dorsal root ganglion neurons in culture also maintains mRNA expression of the neural
cell adhesion molecule L1, survival of Schwann cells, and axon fasciculation, all of which are important for growth cone guidance and extension (Itoh et al., 1995). In this study, low frequency electrical stimulation may have allowed the embryonic neurons to send out growth cones and reach a substrate for neurite attachment faster at first, enabling more axons to successfully grow from the transplant and become myelinated (Fig. 3.2E). This is supported by similar numbers of unmyelinated axons in MG nerves from the Low frequency and No stimulation groups but higher number of myelinated axons following Low frequency stimulation. Brief electrical stimulation of the proximal stump of transected or crushed nerves also increased the number of motoneurons that initiated axon regeneration across the suture line or sped up the return of motor recovery (Al-Majed et al., 2000a; Pockett and Gavin, 1985).

More functional muscles following one hour of transplant stimulation

There were more functional muscles in groups that received a cell transplant and one hour of stimulation compared to groups that received only 3 minutes of stimulation or no stimulation at all (Fig. 3.3). The longer duration of stimulation may have affected transcription of axon-enhancing genes such as the one encoding BDNF (Fields et al., 1997). Stimulation activates several signaling pathways that can elevate CREB phosphorylation, an upstream regulator of BDNF gene transcription. In vivo data suggest that at least 30 minutes of electrical stimulation is needed to reach maximal levels of CREB phosphorylation (Fields et al., 1997). Stimulation inactivates the protein phosphatase, calcineurin, which may prolong CREB phosphorylation levels and targeted gene transcription further (Bito et al., 1996). These results are consistent with an increase
in the number of muscles that were reinnervated and functioned when embryonic neurons were stimulated for one hour. Three minutes of stimulation was presumably insufficient to activate signaling pathways that enhance axon regeneration.

**Muscles were reinnervated from the cell transplant which reduced muscle atrophy**

Cell transplantation resulted in muscle reinnervation, which was clearly beneficial to muscle fiber area, even though brief electrical stimulation of the transplants did not reduce muscle atrophy further (Fig. 3.4). Restoration of muscle fiber areas to 36-44% of uninjured values, across groups, likely reflected the return of neurotrophic support to muscle. No further increase in muscle fiber area with brief transplant stimulation may represent a ceiling effect. Without muscle activity or sufficient weight bearing, neurotrophic support alone cannot maintain muscle fiber area (Pierotti et al., 1991; Tidball et al., 1998; Wang et al., 2006). Similarly, only 33-48% of muscle fibers were reinnervated (Fig. 3.4E). Since axons can sprout within muscle to reinnervate 4-5 times their usual number of muscle fibers (Thompson and Jansen, 1977), and axon counts across groups averaged from 24% to 82% uninjured, it is conceivable that all muscle fibers could have been reinnervated. The incomplete reinnervation observed here may be explained by low axon counts in some cases (Fig. 3.2), a potential mismatch between the neurotransmitters released by regenerating axons (e.g. glutamate) and the muscle target (acetylcholine, Francolini et al., 2009) although motoneurons retrogradely labeled from reinnervated muscles were ChAT-positive (Casella et al., 2010), collapsed intramuscular sheaths following long-term denervation (Fu and Gordon, 1995), the presence of
immature neuromuscular junctions, which are common in our model (Grumbles et al., 2012), and/or neuromuscular junctions that were non-functional.

**Axon regeneration affected muscle force**

Increased axon regeneration by brief low frequency transplant stimulation increased maximum force in MG muscles due to reinnervation of more muscle fibers and larger fiber areas (Fig. 3.5). The effects of transplant stimulation on maximum muscle force were likely mediated via the significant improvements in axon regeneration (Fig. 3.2) because the brief electrical stimulation was delivered to transplants of dissociated cells and did not evoke EMG in the denervated target muscles. Myelinated axon counts were also higher in the MG nerves of the Low frequency versus the No stimulation groups but the unmyelinated axons counts and myelin thickness were similar for these two groups. Differences in maximal muscle forces did not reflect variations in neuromuscular junction function because EMG area was maintained with repeated stimulation but the force declined by a similar amount across groups. Studies at the motor unit level could distinguish whether the enhanced reinnervation represented the formation of more motor units, higher unit innervation ratios, or both possibilities. Irrespective of the underlying mechanisms for the improved force, brief electrical stimulation of the transplant improved both the number of functional muscles and the extent of muscle function. This increase in force with brief transplant stimulation is likely representative of other target muscles too since axons grew equally well into different nerve branches (Figs. 3.2F, G). Better results for the Low and High frequency groups versus the Intermittent group may reflect that immediate early gene expression is increased when there are short intervals...
between trains of stimuli and thus between electrically induced calcium transients (Fields et al., 1997). Poorer results for the Scrambled and Duration control groups suggest that an irregular pattern of stimulation or a short period of stimulation induces calcium transients that are suboptimal for axon-enhancing gene expression.

**Electrical stimulation did not improve motoneuron survival**

Although the current study yielded promising results in terms of axon regeneration and muscle function with brief electrical stimulation of the transplant, motoneuron survival was similar across groups (Fig. 3.6D). These results contrast with an earlier study where we have shown that motoneuron survival was increased when ventral cell preparations were stimulated at 20 Hz for 1 hour (Grumbles et al., 2013). Neural and glial progenitors were abundant in ventral cell preparations. Embryonic motoneurons depend on multiple trophic factors for survival and axon growth, some of which are produced by astrocytes (Eagleson et al., 1985; Taylor et al., 2007). However, astrocytes were uncommon in our purified transplants so are unlikely to have altered motoneuron survival. It is possible that ancillary progenitors in ventral spinal cord preparations are recruited to assist in motoneuron survival. Further studies will be necessary to determine what factors limit motoneuron fate in these transplants.

**Functional implications**

This study demonstrated that brief transplant stimulation at low frequency increased axon regeneration significantly, resulting in increased muscle force due to better muscle reinnervation and reduced muscle atrophy. Restoring muscle fiber area up to 44% of
control values could have multiple benefits. In people with spinal cord injury larger muscles may reduce skin breakdown by providing a cushion between bone and skin, enhance body image, and boost self confidence. Reinnervation of muscle also lowers excitation threshold and opens the opportunity for functional electrical stimulation. Patterned stimulation can provide many useful functions including control of hand grasp, bladder or bowel control (Peckham and Knutson, 2005), which may relieve dependence on caregivers, social and economic costs. In the future, brain computer interfaces may also allow direct control of innervated muscle and movement without involvement of a damaged spinal cord (Andersen et al., 2010; Hochberg et al., 2006; Nicolas-Alonso and Gomez-Gil, 2012).
Electrical Stimulation of Transplanted Neurons Increases the Number of Reinnervated Motor Units

Background

Motoneuron death occurs with spinal cord injury, 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 with denervation, become less excitable, fatigable, and contract and relax more slowly (Ashley et al., 2007a; Finol et al., 1981; Gundersen, 1985; Gutmann, 1948; Hennig and Lømo, 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 atrophy following complete denervation is to electrically stimulate muscle. When stimulation was initiated immediately after denervation and was delivered every day or week day, as in small rat, rabbit and cat muscles, muscle properties recovered to 37-85 % uninjured values (Dow et al., 2005; Hennig and Lømo, 1987; Salmons and Vrbová, 1969; Westgaard and Lømo, 1988). However, when denervated human muscle was stimulated 6-12 months after spinal cord injury and continued for 1-8 years, increases in muscle fiber area were focal.
Large muscle fibers were restricted to the vicinity of the stimulating electrodes (Mödlin et al., 2005). Fibers further from the electrodes remained 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. Neural progenitor cells, bone marrow stromal cells and embryonic stem cells have been transplanted into the injured spinal cord to replace motoneurons in animal models (Abematsu et al., 2010; Deshpande et al., 2006; Li et al., 1997; Reier, 2004; Sandner et al., 2012; Wyatt et al., 2011). When recovery of muscle function was tested, it was poor. Motoneuron survival was low, axon regeneration was obstructed by glial scar, cysts and/or deterioration of peripheral nerves, and axons had to grow long distances to reach target muscles (Kwon et al., 2010).

A muscle has been placed near the spinal cord to shorten the distance axons have to grow to reinnervate muscles (Clowry et al., 1991), but this eliminated the usual muscle action. 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 (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 hour; 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., 2002; Hanson et al., 1998; Perez-Garcia et al., 2008). An important question is whether my stimulation protocol was optimal. In this study, I have electrically stimulated embryonic motoneurons
at various frequencies for up to one hour immediately after transplantation in nerve. The
transplant medium contained neurotrophic factors shown to improve motoneuron survival
and axon growth (Grumbles et al., 2009). My first aim was to determine whether brief
electrical stimulation of the transplant increased the number of reinnervated motor units
in ankle extensor muscles. Second, did the effects on motor units depend on stimulation
frequency, pattern, duration and/or the number of pulses? Third, do the contractile
properties of the reinnervated, denervated and uninjured medial gastrocnemius muscles
differ? Functional behaviors can be restored to innervated muscles using patterned
electrical stimulation whereas muscle denervation is an exclusion criterion.

Materials and methods

Animals

All procedures performed on animals were approved by the University of Miami Animal
Care and Use Committee and adhered to the animal care and use guidelines of the
National Institutes of Health. Ventral spinal cord cells dissociated from day 14-15
embryos were transplanted into three month old inbred Fischer rats (mean weight:163±2
g; Harlan Laboratories, Indianapolis, IN) to avoid the need for immunosuppression.

Experimental design

The left sciatic nerve was transected to denervate many hind limb 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
hour, immediately after transplantation, to test whether this brief intervention altered the number of reinnervated motor units in ankle extensor muscles (see, Stimulation intervention; Fig. 4.1). Reinnervated motor unit numbers and muscle properties were assessed physiologically 10 weeks after transplantation and electrical stimulation.

Muscle denervation

Animals were anesthetized with an intraperitoneal injection of Nembutal (40 ml/kg IP, Akorn Inc, 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 and to expose the sciatic nerve. Two sutures were tied to the sciatic nerve, ~4 mm apart, and ~15-20 mm proximal to where the tibial nerve entered the gastrocnemius muscles. A 2 mm section of nerve was removed between the sutures. These procedures produced denervation of multiple hind limb muscles. The proximal end of the sciatic nerve was tied to the adductor femoris muscle, and remained there until sacrifice, 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 et al., 1999). Briefly, the ventral spinal cord was dissected from day 14-15 embryos (E14-15) in calcium and magnesium-free phosphate buffered saline (PBS). The cord tissues were incubated in trypsin solution for 15 minutes, washed with Leibowitz-15 (L15) solution three times (Invitrogen, Carlsbad, CA), put in
Figure 4.1 Stimulation treatments. Immediately after transplantation, the embryonic cells were stimulated electrically using 1 of 6 protocols, termed High frequency [20 Hz for 1 hour (h)], Low frequency (1 Hz for 1 h), Scrambled (11-30 Hz for 1 h, mean frequency 20 Hz), Intermittent (20 Hz for 6 seconds every 2 min for 1 h), Duration control (20 Hz for 3 min), No stimulation. Animals in the No Cells and Uninjured group also received no stimulation. Black bars indicate periods of transplant stimulation. Horizontal lines indicate delivery of no stimulation. The maximum rest time (MRT) and total number of pulses (Pulses) are indicated for each group (right).
deoxyribonuclease (DNase) solution for 15 minutes, and triturated in bovine serum albumin (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 913g for 15 minutes. Large cells were collected between the Optiprep and media (~73% motoneurons, using Islet-1 to mark embryonic motoneurons; Grumbles et al., 2012), suspended in 4% BSA, centrifuged for 10 minutes at 230 g, and re-suspended in L15 solution. Neurotrophic factors were added to the medium since they increased motoneuron survival in vitro (Hanson et al., 1998) and in our in vivo model (Grumbles et al., 2009). Factors included brain-derived neurotroph factor (BDNF, 10 ng/ml), ciliary neurotrophic factor (CNTF, 10 ng/ml), GDNF (10 ng/ml), HGF (10 ng/ml), IGF-1 (10 ng/ml), and forskolin (10 µM). Cells were counted using Trypan Blue staining and a hemocytometer.

**Cell transplantation**

Embryonic cells were transplanted into the tibial nerve of anesthetized animals one week 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 media were injected into the distal tibial nerve stump, 10-15 mm from where the tibial nerve entered gastrocnemii muscles. Animals in the No Cells group only had 5 µl of medium injected into the tibial nerve without cells to control for the effects of cell transplantation.
Stimulation intervention

Immediately after transplantation, the tibial nerve was laid on a pair of electrodes for stimulation at an intensity that was supramaximal for all axons (3 V, 100 µs duration pulses, Al-Majed et al., 2000a; S48 or S88 stimulator, Natus Neurology, Warwick, RI). The experiment involved 8 groups of animals (Fig 4.1). Animals in the High frequency group received continuous stimulation at 20 Hz for 1 hour (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., 2000a). For this group, the maximum rest time (MRT) during the stimulation intervention was the same as the inter-pulse interval (50 ms). Animals in the Low frequency group received continuous stimulation at 1 Hz for 1 hour (n=3,600 pulses, MRT: 1000 ms) because this stimulation sped up the return of motor function following nerve crush (Pockett and Galvin, 1985). Animals in the Intermittent group received stimulation at 20 Hz for 6 seconds, every 2 minutes for one hour (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 received continuous stimulation for 1 hour at various frequencies (11-30 Hz) that averaged 20 Hz, but 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 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). Animals in the No Stimulation group had the tibial nerve laid on the electrodes for 1 hour but no electrical stimulation was delivered to
control for the effects of stimulation (MRT: 60 min). The tibial nerve of animals in the No Cells group was positioned similarly on the electrodes for 1 hour after transplantation and also received no stimulation. Data from all groups of animals were compared to that obtained from age-matched uninjured animals.

**Physiological recordings**

**Experimental setup**

Muscle properties and motor unit counts were assessed physiologically 10 weeks after cell transplantation. The transplant, medial gastrocnemius (MG), lateral gastrocnemius (LG) and plantaris (PL) muscles were carefully separated from surrounding tissue without damaging blood vessels. For recording, animals were laid prone on a heating pad to keep body temperature at 37 °C. Skin from the leg was used to form a pool for mineral oil to keep the transplant and muscles moist. Muscle temperature was kept at 35-37 °C using a heat lamp. The knee and ankle were clamped to prevent movement during recordings. Suture was tied to the distal tendons of MG, LG and PL muscles. In turn, each tendon was connected to a transducer (FT03, Natus Neurology, Warwick, RI) to record isometric muscle force. A pair of silver electrodes was laid on the mid belly of each muscle to record electromyographic activity (EMG). The tibial nerve (transplant) was laid on another pair of silver electrodes for stimulation (S88 stimulator). EMG and force were recorded online using a SC/Zoom system (400 Hz and 3200 Hz, respectively; Umeå University, Sweden).
Experimental protocol

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 until maximal twitch force was determined. All subsequent stimulation was delivered at this optimal muscle length. 2) To count the number of reinnervated motor units, stimulation voltage (50 µs pulses) was increased from subthreshold (1 V) to supramaximal intensity (150 V) in 0.1 V, 1 V 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 µs and 10 µs). 3) To determine muscle excitability, 5 single pulses of increasing intensity were delivered (10, 30 and 150 V each for 50 µs, 150 V for 100 µs, 150 V for 1000 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. Contraction were evoked by stimulation at different frequencies (5, 8, 10, 15, 20, 30, 40, 50 Hz for 1 s; 100, 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 minutes (Burke et al., 1973). These data were compared to results from 5 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, 1000 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, medial gastrocnemius was then stimulated directly to examine denervated muscle excitability as described above, the force evoked by stimulation at 40, 50, and 100 Hz and fatigue, both using 150 V and 1000 µs pulses.

**Physiological analysis**

All physiological analyses, including measurement of EMG, force, and timing of events, were completed offline using custom software (Zoom, Umeå University, Sweden).

**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., 2003). 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 digitally subtracted from each other to obtain the force of each motor unit, my measure of motor unit size. 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 1000 µs but the number of responses was 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 to the number of MG motor units to determine the number of axons that successfully made functional connections to 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 Hemotoxylin and Eosin (Electron Microscopy Science, Hatfield, PA), imaged at 40x magnification, and the areas of 500 sampled muscle fibers 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 larger than 95% of the fibers in completely denervated muscles (>498 µm²; Grumbles et al., 2008). Mean muscle fiber area and reinnervation were compared to 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 (version 20, IBM Corporation, 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 indices were analyzed using One-way Analysis of Variance (ANOVA). The MG muscle force evoked at different stimulation frequencies (1-200 Hz), as well as the twitch force elicited in response to different stimulation intensities, were compared across groups using Two-way Repeated Measurement ANOVA. Pearson’s 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, and 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). Significance level was P<0.05.
Results

Continuous transplant stimulation for one hour increases 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. 4.2A-C). In reinnervated medial gastrocnemius (MG), lateral gastrocnemius (LG) and plantaris (PL) muscles, motor unit counts ranged from 0-25, 0-21 and 0-16, respectively (n=76 muscles). In uninjured MG, LG and PL muscles, the number of motor units varies from 85-115, 89-127 and 66-92, respectively (Finkelstein et al., 1991; Hashizume et al., 1988; Nicolopoulos-Strourmaras and Iles, 1983; Novikova et al., 1997). The force, CT and hRT of different motor units also varied within and across muscles. Table 4.1 shows mean motor unit counts by muscle and group.

My results suggest that one hour of continuous stimulation is important for transplanted motoneurons to form motor units. The mean (±SE) number of motor units per muscle ranged from 2±1 to 5±1 across groups (Fig. 4.2D) and was significantly higher for the High frequency (P=0.014), Low frequency (P=0.038) and Scrambled (P=0.012) groups compared to the Duration control group (20 Hz, 3 min). The common feature for the three groups with higher motor unit counts was continuous transplant stimulation for 1 hour. When the data from these three groups were pooled (Cont Stim), the motor unit count per muscle exceeded that for the No Stimulation (P=0.045) and Duration control groups (P=0.004; Fig. 4.2D).
# Table 4.1 Mean (±SE) motor unit count 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>High frequency</td>
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<td>5±1</td>
<td>5±1</td>
<td>15±3</td>
</tr>
<tr>
<td>Low frequency</td>
<td>5±2</td>
<td>3±1</td>
<td>5±2</td>
<td>13±3</td>
</tr>
<tr>
<td>Scrambled</td>
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<td>6±2</td>
<td>4±1</td>
<td>15±4</td>
</tr>
<tr>
<td>Intermittent</td>
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<td>3±1</td>
<td>4±1</td>
<td>10±2</td>
</tr>
<tr>
<td>Duration control</td>
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<td>1±1</td>
<td>4±1</td>
<td>7±2</td>
</tr>
<tr>
<td>No Stim</td>
<td>3±1</td>
<td>3±1</td>
<td>3±1</td>
<td>10±1</td>
</tr>
</tbody>
</table>
Figure 4.2 Motor unit counts and force. Overlay of averaged MG force steps evoked by stimulation of a transplant from the High frequency (A), Scrambled (B) and Duration control (C) groups 10 weeks after cell transplantation. (D) Mean (±SE) motor unit count for MG, LG and PL muscles across groups and when data for the High, Scrambled and Low frequency groups were pooled (Continuous stimulation group, Cont Stim; * P<0.05, one-way ANOVA). (E) Maximum rest time (log_{10} scale) versus mean MG, LG and PL motor unit count across groups (R^2=0.995; P<0.01, Pearson correlation). Data from the No stimulation group were excluded from the correlation. (F) Mean (±SE) motor unit force of MG, LG and PL muscles across groups, expressed relative to the respective uninjured twitch forces. Short group names are used in all figures: No Stimulation, No Stim; Duration control, Dur; Intermittent, Int; Low frequency, Low; Scrambled, Scr; High frequency, High.
The maximum rest time (MRT) was shorter in groups with higher motor unit counts (P=0.002, Fig. 4.2E). Motor unit numbers were highest in the High frequency and Scrambled groups which had the shortest maximum rest times (50 ms and 91 ms, respectively), whereas the unit count was lowest in the Duration control group (MRT: 57 min) and 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 continuous transplant stimulation for one hour with shorter MRT was more important for motor unit formation than the frequency of stimulation or the total number of pulses. Both the Duration Control and High frequency groups received 20 Hz stimulation but there were more motor units in the High frequency group. Both the Duration Control and Low frequency groups received 3600 pulses but there were more motor units in the Low frequency group.

**Brief transplant stimulation improved motor unit counts and muscle force**

The number of reinnervated MG motor units was higher when more axons regenerated from the transplant, even though there were 8-13 times more myelinated axons than motor units (P=0.041, Fig. 4.3A). Higher motor unit counts also resulted in larger muscle fiber areas, and greater reinnervation but the relationships were not significant (P=0.090, Fig. 4.3B; P=0.133, Fig. 4.3C). Higher motor unit counts did increase maximal tetanic forces (P=0.022, Fig. 4.3D), although mean motor unit force was similar across groups (range: 0.79±0.13% to 1.12±0.15% of uninjured muscle twitch force), irrespective of whether the cells were stimulated immediately after transplantation or not (Fig. 4.2F).
Figure 4.3 Correlations between parameters. Mean MG motor unit count across groups as a function of MG myelinated axon count (A, $R^2=0.54$, $P<0.05$), reinnervated muscle fiber area (B, $R^2=0.60$), percentage of reinnervated muscle fibers (C, $R^2=0.69$), and maximum muscle force (D, $R^2=0.77$, $P<0.05$, Pearson correlation).
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. 4.4A) versus uninjured muscles (Fig. 4.4B). When responses to different frequencies of stimulation were normalized to maximal muscle force, reinnervated muscles generated higher relative forces than uninjured muscles between 15 and 50 Hz (P<0.05; Fig. 4.4C). Half maximal force (F50) was also reached at significantly lower frequencies in reinnervated muscles (15±2 to 21±3 Hz across groups) versus uninjured muscles (42±2 Hz, P<0.001). Muscles reinnervated from the transplanted cells had slower twitch contraction times and half relaxation times than uninjured muscles (Fig. 4.4D), across groups (Fig. 4.4E, both P<0.01), one factor that contributed to the greater fusion of forces in response to low stimulation frequencies.

The mean reinnervated muscle twitch force evoked by 10 V (50 µs pulse duration) ranged from 54 ± 14 % to 80 ± 16 % maximal across groups, whereas maximal force was evoked in all uninjured muscles (P<0.05, except for muscles in the Scrambled group, P=0.083). When the stimulation intensity was increased to 30 V, the mean relative twitch forces of reinnervated and uninjured muscles were comparable. The higher stimulation intensities needed to evoke maximal twitch forces in reinnervated versus uninjured muscles likely reflects the lower electrical excitability of smaller diameter axons (Blair and Erlanger, 1933; Moore and Westerfield, 1983). Axons that regenerated from the transplants into the MG nerve had smaller mean diameters (range: from 3.5±0.3 µm to 4.0±0.2 µm across groups) than axons from uninjured animals (10.7±0.2µm).
Figure 4.4 Force gradation and contractile speed. MG forces evoked from a reinnervated (A; Scr group) and uninjured (B) muscle in response to stimulation (150 V, 50 µs) at different frequencies. (C) Force expressed relative to maximal muscle force, averaged by group, plotted in relation to stimulation frequency. (D) MG twitch force evoked from a reinnervated (Scr group) and uninjured muscle, normalized by peak force. (E) Mean (±SE) twitch contraction time and half-relaxation time across groups (Both ** P<0.01, one-way ANOVA). Muscles in the No Cells group remained denervated as no force was evoked by stimulation of the transplant.
**Reinnervated muscles were more fatigue resistant than uninjured muscles**

Evoked force declined less in reinnervated (Fig. 4.5A) versus uninjured muscles (Fig. 4.5B) when trains of pulses were delivered at 40 Hz for 2 minutes. The mean force fatigue indices ranged from 0.53±0.07 (Duration control group) to 0.84±0.02 (High frequency group) for reinnervated muscles, all of which were significantly higher than the value for uninjured muscles (0.23±0.03; P<0.001, Fig. 4.5C). Fatigue indices for EMG area did not differ for reinnervated (range: 1.09±0.09 to 1.28±0.22 across groups) and uninjured muscles (1.01±0.16, Fig. 4.5C). These results suggest there was effective transmission of signals to the sarcolemma and that the reduction in force must reflect impaired processes in the muscle fibers.

**Denervated muscles only generated force through direct high intensity 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 axons (Fig. 3.2E). Thus, these muscles remained denervated. The only way to elicit force was to stimulate these denervated muscles directly (Fig. 4.6). Direct muscle stimulation at 10, 30, and 150 V (50 µs) generated an average force of 0.5±0.2 (<3 mN), 18±2 and 88± 1% maximal force, respectively. Small force increases occurred at higher stimulus intensities (150 V, 100 us pulses, Fig. 4.6A). Maximal force was evoked in response to a train of pulses at 40 Hz (Fig. 4.6B) resulting in a mean twitch to tetanic ratio of 0.66±0.01 for all denervated MG muscles. Denervated muscles were fatigable (Fig. 4.6C). The mean force declined to 25±2% of initial after 2 minutes of direct muscle stimulation at 40 Hz.
**Figure 4.5** Muscle fatigue. MG forces evoked from a reinnervated (A; Scr group) and uninjured (B) muscle at the beginning (solid line) and end (dashed line) of 2 minutes of stimulation (13 pulses at 40 Hz every second, 150 V, 50 µs). (C) Mean MG (±SE) fatigue indices (final/initial values) for EMG area and force across groups. Force fatigue indices for reinnervated muscles were higher than those of uninjured muscles (***, P<0.001, one-way ANOVA).
Figure 4.6 Denervated MG muscle properties. (A) Twitch force evoked when a denervated muscle (No Cells group) was directly stimulated at different intensities (10, 30, 150 V using 50 µs pulses; 150 V, 100 µs). (B) Forces evoked from denervated muscle at 1 Hz and 40 Hz (150 V, 50 µs) and (C) at the beginning (solid line) and end (dashed line) of 2 minutes of stimulation (13 pulses at 40 Hz every second).
Discussion

The main finding of this study is that dissociated neurons transplanted into an adult peripheral nerve responded to brief electrical stimulation, leading to an increase in the number of reinnervated motor units in ankle extensor muscles. The reinnervated motor unit count and muscle force were higher when the stimulation lasted for one hour and the maximal rest time during the stimulation intervention was shorter. These results suggest that patterned electrical stimulation of embryonic neurons, in vivo, has long-term functional consequences on motor unit formation and muscle function.

Electrical stimulation for one hour 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., 2009; Grumbles et al., 2009; Hanson 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, 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.

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). Up regulation of cAMP also increased the number of embryonic motoneurons with axon growth and neurite length (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 (Naeem et al., 2002; Zurn et al., 1996). This mechanism is one possible way to explain the stimulation-specific increase in both axon (Fig. 4.3A, 3.2E) and motor unit counts (Fig. 4.2D). Other studies show that electrical stimulation of the proximal stump of 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., 2000a; Pockett & Gavin, 1985) by increasing BDNF expression in neurons (Al-Majed et al., 2000b; English et al., 2006). 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 axons 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
minutes was required for sustained levels of phosphorylated CREB and for decreased
dephosphorylation by phosphatase-I (Bito et al., 1996; Liu and Graybiel, 1996; Lyons
and West, 2011). Our results show continuous stimulation of transplanted neurons for one
hour increased the number of reinnervated motor units beyond that measured after 3
minutes of stimulation or no stimulation (Fig. 4.2E). Second, longer rests between trains
of stimuli resulted in lower levels of phosphorylated CREB and immediate early gene
(cfos) expression (Fields et al., 1997). I had higher reinnervated motor unit numbers in
groups where the maximal rest within the stimulation treatment was shorter (Fig. 4.2D,
E). 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 myelinated axon counts improve motor unit formation and muscle force
Groups with more reinnervated MG motor units had stronger MG muscles (Fig. 4.3D)
without significant increases in muscle fiber size (Fig. 4.3B), reinnervation (Fig. 4.3C,
3.5) or motor unit force (Fig. 4.2E). These results suggest that a higher number of
myelinated axons with the ability to form functional motor units (Fig. 4.3A) was the main
long-term effect of electrical stimulation. Brief electrical stimulation may also have
induced 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 µm to 23.0±1.2 µm compared to 30.4±3.5 µm for L4/L5 spinal
motoneurons (Kanda and Hashizume, 1998). Our reinnervated muscles were weak, slow,
and fatigue resistant (Fig. 4.3D, 4.5, 4.6), properties that are characteristic of slow,
fatigue resistant motor units (Botterman et al., 1985; Kernell et al., 1983). Since fibers reinnervated from embryonic motoneurons largely become Type IIA and I (Thomas et al., 2003), these results 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; Foehring et al., 1987; O'Donovan et al., 1985).

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. 4.3C) even though myelinated axon counts exceeded the number of reinnervated motor units (Fig. 4.3A). My data suggest there are an average of 1179±34 motoneurons in the uninjured tibial nerve, similar to published data (range: 962-1077, Todorova and Rodziewicz, 1995), but 82±1 of these axons enter the MG nerve branch (7%). Given that 385±107 transplanted motoneurons survived at 10 weeks, and myelinated axons were proportionally distributed across all nerve branches (Fig. 3.2 F and G), 7% of these axons would be expected to enter the MG nerve and be available to form motor units (n=27). MG axon and motor unit numbers ranged from 1-242 and 0-25, respectively, and multiple factors may underlie this difference. For example, axons from non-cholinergic neurons present in our transplants may fail to innervate muscle (Grumbles et al., 2012). The neuromuscular junctions do function reliably, shown by the maintenance of EMG area in response to repeated stimulation (Fig. 4.5C). Nevertheless, our earlier data shows polyneuronal innervation in our reinnervated muscles after transplantation, but the evoked EMG was of consistent amplitude, suggesting stable muscle activation (Grumbles et al., 2007) rather than
inconsistent activation of various neuromuscular junctions. In the long-term, regenerating axons are not forced to innervate the same muscle fibers in our model because some fibers usually remain denervated. Thus, over time, polyinnervation may be eliminated (Thomas et al., 2000). In addition, EMG amplitude did not increase markedly when stimulation was delivered at higher frequencies, even when only one motor unit was excited. These results suggest that temporal summation of subthreshold potentials in response to different frequencies of stimulation, as seen in neonatal rats (0-1 weeks after birth; Bennett and Pettigrew, 1974; Ijkema-Paassen et al., 2001; McArdle, 1975), was not a major factor 10 weeks after cell transplantation. Even so, some junctions may be immature and non-functional, 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 (Pierotti et al., 1991). The increases in CT and hRT with reinnervation (Fig. 4.4E) may also arise from reduced use, possibly from slowing of calcium uptake (Duchateau and Hainaut, 1987; Taylor et al., 1997). It may also reflect a change to type I and IIA fibers (Foehring et al., 1986; Thomas et al., 2003), and/or result from greater muscle compliance after reinnervation (Huyghues-Despointes et al., 2003a, b). Both the slow CT and hRT, as well as the slight increase in twitch-tetanic force ratios contributed to the greater force fusion at a given stimulation frequency, and achievement of half maximal force at 17±1 Hz versus 42±2 Hz in reinnervated versus uninjured muscles (Fig. 4.7C).
Figure 4.7 Muscle properties. Mean (±SE) (A) force (normalized to the maximum force of each muscle), (B) twitch-tetanic force ratio, (C) F50, and (D) force fatigue index (C) for uninjured (Uninj), reinnervated (Reinn) and denervated muscles excited via nerve (No cells, nerve) or direct muscle stimulation (No cells, muscle).
**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. 4.6), confirming previous data (e.g. Ashley et al., 2007a; Finol et al., 1981; Gundersen, 1985; Gutmann and Young, 1944; Kotsias and Muchink, 1987; Schmalbruch et al., 1991). In contrast, reinnervated muscles could be excited by nerve stimulation, produced higher forces (relative to maximum) at 10 and 30 V (Fig. 4.7A), had low twitch/tetanic ratios (Fig. 4.7B), reached half maximal force at low stimulation frequencies (Fig. 4.7C) and were fatigue resistant (Fig. 4.7D). These differences illustrate why denervation is an exclusion criteria for functional electrical stimulation applications. Not only is little force evoked in denervated muscles at low stimulation intensities (Fig. 4.7A), one high intensity, wide duration pulse evoked 66±1 % of maximal force (Fig. 4.7B), leaving limited scope to grade force by changing pulse width, intensity or frequency. Denervated muscles were also fatigable (Fig. 4.7D). Besides these less than optimal contractile properties, the high stimulation intensities needed to excite denervated muscles would raise the power requirements of any stimulation device, 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. Maximal twitch forces were gradable at relatively low stimulation intensities by changing pulse amplitude or duration (Fig. 4.7A) as nerve is more excitable than muscle (Mortimer, 1981). Recruitment of all axons by a single stimulus evoked 26±2 % of maximal force (Fig. 4.7B), leaving an average of 74 % of force to be generated by modulating stimulation
frequency (Botterman et al., 1986). Further, half maximal muscle force was reached at 17±1 Hz (Fig. 4.7C). The use of fewer pulses will reduce fatigue (Fuglevand et al., 1999; Thomas et al., 2003). Even though reinnervated MG muscles often only produced a fraction of the force of uninjured muscles, other synergists extend the ankle. When activated together by transplant stimulation, these muscles may produce sufficient force for locomotion (~5% maximal; Ichihara et al., 2009; Jung et al., 2009; Thomas et al., 2010) or to move a joint against gravity (9% force; Needham-Shropshire et al., 1997).

The fatigue resistance of reinnervated muscles (Fig. 4.7D) 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. 4.2D). Higher motor unit numbers increase the number of axons to stimulate, gradation of force, and force production.
Conclusions and Discussion

This study showed that brief electrical stimulation of embryonic neurons transplanted in peripheral nerve for just one hour had long-term functional consequences. Five main conclusions can be drawn from this research.

1. One hour of low frequency stimulation (1 Hz) increased the number of myelinated axons in the tibial nerve (Fig. 3.2E) but the myelinated axon counts exceeded the number of reinnervated motor units (Fig. 4.3A). Muscle fiber areas of non-functional MG muscles averaged 18±1 % uninjured values whereas denervated muscle fiber areas were 9±1 %, suggesting some neuromuscular junctions may be immature and non-functional. Formation and maturation of neuromuscular junctions is activity-dependent (Fukazawa et al., 2013; Johnson and Connor, 2011). Chronic stimulation of the reinnervated muscles may therefore increase muscle function (Kernell et al., 1987a, b; Hennig and Lømo, 1987). Another possibility is that incomplete differentiation of motoneurons in nerve means some neurons are unable to reinnervate muscle (Reimer et al., 2008), limiting function. Single or double transduction of genes that control late stage motoneuron differentiation may be needed to overcome a lack of appropriate extrinsic cues (Dalla Torre di Sanguinetto et al., 2008; Roybon et al., 2008; Wijeyekoon and Barker, 2009) and to improve muscle reinnervation.
2. Continuous stimulation for one hour that involved shorter rests increased the number of reinnervated motor units in ankle extensor muscles (Fig. 4.2E). These findings suggest that activity-based increases in motor unit numbers depend on the temporal pattern and duration of signaling. Higher motor units counts with brief stimulation augment the increase in motor unit force seen when neurotrophic factors are added to the transplant medium (Casella et al., 2010). Chronic support may improve muscle reinnervation further (e.g. slow release of neurotrophic factors from nanoparticles; Yurek et al., 2009).

3. Brief electrical stimulation of the transplanted cells did not change motoneuron survival. However, we have previously shown that electrical stimulation increased survival of motoneurons in transplants of ventral spinal cord cells (Grumbles et al., 2013). The ventral spinal cord cell preparation included motoneurons and glial progenitor cells whereas the present transplants were purified for motoneurons. These findings suggest that glial cells play an important role in survival of embryonic motoneurons, which involves mutual interactions, possibly by release of trophic factors (Eagleson et al., 1985; Taylor et al., 2007).

4. Higher numbers of reinnervated motor units resulted in stronger muscle forces but there was still chronic denervation of some muscle fibers. MG muscle force reached 30% uninjured in some animals so there is clear potential for improvement. Survival of larger motoneurons with higher innervation ratios (Bodine et al., 1987; Tötösy de Zepetnek et al., 1992a) may enhance muscle reinnervation and force. Given that our reinnervated
muscles are inactive, chronic stimulation against resistance may double muscle fiber size and force (Kernell et al. 1987a, b; Kim et al. 2007; Pierotti et al. 1991).

5. Reinnervated muscles could be excited by nerve stimulation. Only direct muscle stimulation induced contractions in denervated muscles. Even then, high twitch-tetanic force ratios and muscle fatigue left little opportunity for gradation of force by frequency or over time. Thus, it is not surprising that individuals with muscle denervation are excluded from functional electrical stimulation applications.

Finally, what level of motoneuron survival and reinnervation is needed for meaningful function? During human locomotion, EMG levels in leg muscles only average 5% maximal (Klein et al., 2010; Thomas et al., 2010). In people with spinal cord injury, only 9% of control force is needed to move a joint against gravity (Needham-Shropshire et al., 1997). Signals that are 5% maximal in synergists like MG, LG, and PL may be adequate for ankle movement when their actions are combined (Figure 4.3D; Jung et al., 2009; Thota et al., 2005). Further, spinal cord injury in humans usually involves focal contusion (Bunge et al., 1997). In our model, one transplant of neurons causes reinnervation of multiple muscles (Fig. 3.2F, G). This approach may allow more comprehensive control of a joint using patterned electrical stimulation (Peckham and Knutson, 2005), brain computer interfaces (Hochberg et al., 2012), and/or optical signals (Boyden et al., 2005; Llewellyn et al., 2010).
References


