

MOTOR FUNCTION RESTORATION: THERAPEUTIC APPROACHES TO
DENERVATION PATHOLOGIES

by

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Dedication

Learning is a long intricate process, one that requires an orchestrated elaboration of synapses among an already sophisticated neuronal network. I would like to dedicate this thesis to everyone who has contributed to this learning process, might it be by fostering sprouting or pruning.

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Abstract

Completely denervated muscles, as seen after motoneuron diseases such as amyotrophic lateral sclerosis, or after peripheral nerve and spinal cord injuries, do not respond to electrical stimulation and are thus not amenable for neural prostheses. Restoration of motor function requires either a timely return of innervation or an alternate approach to control contraction despite denervation. To do so, we first focused on transplantation of embryonic stem cell-derived motoneurons after prolonged denervation and followed by developing a model to optically control muscle contraction when reinnervation is not possible.

For transplantation, embryonic stem cell-derived motoneurons were injected into the distal segment of the transected murine tibial nerve after prolonged denervation ranging from one to eight weeks. Success of reinnervation by transplanted motoneurons partially restored muscle force but decreased as the denervation period lengthened. Furthermore, teratocarcinomas developed from a residual multipotent cell population found despite neural differentiation. Tumours could be prevented by pre-transplant treatment with a DNA alkylating agent without compromising innervation from transplanted motoneurons.

For optical control of contraction, we generated a transgenic mouse model expressing channelrhodopsin-2/H134R along the sarcolemma and T-tubules of skeletal muscle fibres. This model allowed optical control of contractions with forces comparable to those generated by nerve stimulation. Varying light pulse intensity, pulse duration and pulse frequency permitted to reproduce normal force generation patterns.

Finally, we encountered a series of transplants that generated self-organized circuits resulting in spontaneous muscle contractions and evoked episodes of contraction bursts upon stimulation. This circuit was glutamate dependent and was modulated by inhibitory and muscarinic inputs.

Despite differentiation, embryonic stem cell-derived motoneurons carry a risk of tumorigenicity. Pre-transplantation treatment with an anti-mitotic agent leads to survival and functional muscle reinnervation if performed before eight weeks of denervation. However, differentiation of stem cells towards a ventral spinal neuronal phenotype results in a mixed population enriched in motoneurons that, once transplanted in an environment disconnected from the spinal cord, forms variable degree of self-assemble microcircuits resulting in uncontrolled muscle contractions. An alternative for motor function restoration in permanently denervated muscles can be from incorporation of channelrhodopsin-2 in muscle fibers.

List Of Abbreviations Used

5-HT: serotonin
ACh: acetylcholine
AChR: acetylcholine receptor
AHP: after-hyperpolarization potential
ALS: amyotrophic lateral sclerosis
APV: DL-2-amino-5-phosphonovaleric acid
ChR: channelrhodopsin
ChR2: channelrhodopsin-2
CNQX: 6-Cyano-7-nitroquinoxaline-2,3-dione
CNTF: ciliary neurotrophic factor
CSA: cross-sectional area
DA: dopamine
EB: embryoid body
EC: embryonal carcinoma
EC: excitation-contraction (coupling)
EMG: electromyogram
ES: embryonic stem (cells)
ESCMN: embryonic stem cell-derived motoneuron
FACS: flow assisted cell sorting
GDNF: glial-derived neurotrophic factor
GFP: green fluorescent protein
HBSS: Hank's basal salt solution
iPS: induced pluripotent stem (cell)
LED: light emitting diode
MG: medial gastrocnemius
MN: motoneuron
MND: motor neuron disease
MU: motor unit
NMDA: N-methyl-D-aspartic acid
NMJ: neuromuscular junction
NPC: neuroprogenitor cell
NSPC: neural stem / progenitor cell
PGC: primordial germ cell
PNI: peripheral nerve injury
RA: retinoic acid
SC: Schwann cell
SCI: spinal cord injury
SD: standard deviation
Shh: sonic hedgehog
SOD1: superoxide dismutase 1
SSEA-1: stage-specific embryonic antigen 1
TPNI: traumatic peripheral nerve injury
TTX: tetrodotoxin

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Life is full of surprises, some good, others not so. As angering and frustrating those can be sometimes, life never stop surprising with a turn of events always for the best. Indeed, life will always find a way to sort things out. The real test is one of patience. By waiting long enough, a solution always comes about.

So I waited, waited, and waited until this thesis had written itself. Truth be told, I eventually realized I had to compose it myself. Indeed, life is full of surprises.

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Life is indeed full of surprises.

Chapter One: Introduction

Motor Neuron Diseases

Neuropathic disorders are composed of disorders of the peripheral nerves, named neuropathy, and of neuronal cell body, termed neuronopathy. Motor neuron diseases (MND) are neuronopathies affecting both cortical neurons and spinal motor neurons (Barohn and Amato, 2013). MNDs are motor syndromes without sensory involvement, of insidious onset, chronically progressive and exist as a spectrum of presentations from pure lower MN to pure upper motor neuron with a mixed presentation spanning in between (Statland et al., 2015). MNDs are classified as hereditary or sporadic and by the degree of upper versus lower motor neuron involvement. Ten clinical patterns have been reported with amyotrophic lateral sclerosis (ALS) being classically a mixed upper and lower motor neuron presentation, whereas spinal muscular atrophy is a pure lower motor neuron presentation pattern (Barohn and Amato, 2013).

Amyotrophic Lateral Sclerosis

ALS is a progressive disease invariably fatal secondary to paralysis of respiratory muscles with a median time from diagnosis to death of 2.5 to 3 years (Chancellor et al., 1993; del Aguila et al., 2003; Traxinger et al., 2013). ALS has an annual incidence of 1.52 per 100 000 person-year (Statland et al., 2015), with an average age of onset in the sixth decade and an average time from symptom onset to diagnosis of 10.8 to 16.9 months (Statland et al., 2015). The classic presentation is an insidious onset of weakness followed by progressive worsening demonstrating both upper and lower motor neuron findings on examination. ALS remains a clinically diagnosed disease based on the updated Awaji criteria (de Carvalho et al., 2008); ancillary testing being used to rule out ALS-mimicking conditions. Negative prognostic factors

conveying a faster progression include bulbar onset of symptoms, older age at onset of symptoms, shorter duration of symptoms and reduced force vital capacity (Statland et al., 2015).

The majority of ALS cases are sporadic, with only 10% being familial. Although familial ALS syndromes are less common, together they provide opportunities to develop pathophysiological models in order to understand the process underlying the preferential demise of motor neurons and MN subclasses (Hegedus et al., 2008). The microscopic hallmark feature of ALS is the presence of ubiquitinated inclusion bodies within pyramidal Betz cells, and spinal cord and brainstem motor neurons. Many ALS models revolve around mutated proteins found in these inclusion bodies or involved in protein trafficking and regulation: superoxide dismutase (SOD1), TAR DNA-binding protein (TDP-43) and fused in sarcoma / translocated in sarcoma (FUS-TLS). Recently, expanded hexanucleotide repeats (C9ORF72) were found to be the most common cause of ALS accounting for 30% of familial and 5% of sporadic cases (DeJesus-Hernandez et al., 2011; Renton et al., 2011).

The only approved therapy of ALS is riluzole, hypothesized to decrease glutamate toxicity of motor neurons through a variety of mechanisms (Bellingham, 2011). Survival is prolonged by about three months (Miller et al., 2012). Many trials and approaches have been or are undergoing investigations. A phase two trial for intrathecal transplantation of fetal human stem cells is underway after safety was demonstrated in a phase one study (Feldman et al., 2014). Genetic therapy by intrathecal administration of antisense oligonucleotides targeting SOD expression is expected after proof of safety and tolerability in a pilot human study (Miller et al., 2013).

Neurological Trauma

Spinal Cord Injury

Spinal cord injury (SCI), either traumatic or atraumatic (e.g. vascular or post-infectious injuries), disturbs normal sensory, motor, and autonomic functions debilitating patients physically, psychologically and socially (Sekhon and Fehlings, 2001; Furlan et al., 2011; Noonan et al., 2012). Spinal cord injury affect 41 individuals per million of population per year, cumulating in Canada to a prevalence of 2 525 individuals with spinal cord injury per million of population, or about 90 000 individuals, with the majority being young males (Noonan et al., 2012; Singh et al., 2014). An increasing incidence among the older population has also been identified (Pirouzmand, 2010; Knutsdottir et al., 2012; Singh et al., 2014; Ahn et al., 2015). The long term loss of function results in a life-time socio-economic burden estimated to range from \$1.5 million for paraplegics to \$3 million for tetraplegics (Krueger et al., 2013), without accounting for decades of income loss in most individuals.

Spinal cord injury damages both grey and white matter with the disconnection of white matter pathways, ascending and descending, being responsible for the majority of loss of function below the level of injury. Current spinal cord therapies focus on preventing secondary injury post trauma in order to salvage neural tissue. The loss of grey matter prevents any return of neurological functions at the level of injury. Recovery of functions following loss of grey matter would require regeneration of lost neurons and proper synaptic connections. As adult mammalian neurons have a limited ability for regeneration (Cajal, 1959), therefore cell transplantation therapy is an appealing option to restore functions (Tetzlaff et al., 2011).

Peripheral Nerve Injury

Epidemiology

Most peripheral nerve injuries (PNI) are due to motor vehicle trauma and less so to penetrating trauma. About 2 to 3% of admissions to major trauma centers result from PNIs and in general

involves the upper limb. An additional equal number of plexus and root injuries are seen, culminating to 5% of traumas (Selecki et al., 1982; Noble et al., 1998). Peripheral nerve traumas are much more common in wartime and in fact most knowledge on PNI has been acquired from surgical practice during major wars (Robinson, 2000). Peripheral nerve injuries have a significant socio-economical burden, preventing return to work from a protracted and often meager return of function.

Classification

Traumatic peripheral nerve injuries (TPNI) are classified based on the Seddon (Seddon, 1975) or the Sunderland (Sunderland, 1978) classifications. The Seddon classification is most commonly used in the literature and divides injuries between neurapraxia, axonotmesis and neurotmesis. Neurapraxia is considered a mild injury with temporary loss of function, motor and sensory, from focal demyelination or ischemia causing a conduction block. Neurapraxia does not demonstrate Wallerian degeneration. Recovery occurs between hours to months. Muscles are normally spared from pathological changes except for the onset of disuse atrophy from injuries causing a prolonged conduction block (Fowler et al., 1972).

Axonotmesis reflects a destructive injury to the axon leading to Wallerian degeneration. By definition the epineurium remains intact. The Sunderland classification further sub classifies axonotmesis into an isolated axonal disruption, an axonal and endoneurial injury or an axonal, endoneurial and perineurial injury. Although this classification is mostly anatomical and cannot be differentiated clinically in the acute settings, the outcomes are very different. A pure axonal injury with intact perineurium is best exemplified as a crush injury where recovery is generally complete except if the injury is proximal enough that return of innervation would take more than 12 to 18 months (Robinson, 2000). Destruction of the endoneurium disrupts the basal lamina essential for directing regenerating axons resulting in misdirection and thus results in a guarded

prognosis (Al-Majed et al., 2000). Disruption of the perineurium causes internal disorganization of guiding connective tissue and scarring, both associated with poor prognosis (Siironen et al., 1992). Neurotmesis is a complete transection of the nerve with disruption of the epineurium. These injuries do not spontaneously recover and prognosis despite surgical repair remains guarded, particularly for proximal injuries.

Pathobiology Of Nerve Injury

Most knowledge acquired on the pathophysiology of PNI comes from animal models, primarily from the rat. Any injury or pathology affecting the MN cell body or its axon leads to denervation. An axonal injury causes rapid changes: leakage of intra-axonal fluid, swelling of distal nerve segments, disappearance of neurofibrils, and fragmentation of axon and myelin. The proximal nerve stump sprouts within 24 to 36 hours. SCs proliferate upon loss of axonal contact forming the Bands of Büngner and phenotypically transform to become growth-supportive instead of myelinating (Navarro et al., 2007). Digestion of fragments is complete by day eight. Axons will take eight to fifteen days to bridge the injury site before continuing growth at a rate of 1 to 3 mm/day (Sunderland, 1978; Buchthal and Kühl, 1979; Giuffre et al., 2010).

Recovery of function is progressive, first with resolution of conduction block, muscle fibre hypertrophy in partial lesions, distal axonal sprouting, and axonal regeneration from the site of injury (Robinson, 2000). Axonal sprouting at the neuromuscular junction (NMJ) occurs within four days (Hoffman, 1950) and guide regenerating axons to adjacent denervated endplates (Reynolds and Woolf, 1992; Son and Thompson, 1995). However, early recovery of force in humans is due to improved synchronization of motor unit firing as opposed to sprouting (Milner Brown and Stein, 1975; Moritani and deVries, 1979).

Growth-supportive capacities of Schwann cells (SC) progressively decline, down-regulating the expression of cell adhesion molecules by four weeks (Li et al., 1997), trophic receptors by eight weeks (You et al., 1997) and trophic factors by 12 weeks following injury (Höke et al., 2002; Furey et al., 2007). This process results in a progressive failure of axonal regeneration (Sulaiman and Gordon, 2000). After prolonged denervation of more than five weeks, processes of terminal SCs start to retract, decreasing the capacity for innervation by incoming motor axons (Kang et al., 2014). Realistically, recovery depends on the injury grade, amount of scarring, the gap length between the nerve ends and the age of the patient. However, despite proper axonal regrowth, proximal injuries such as those involving the brachial plexus, are not expected to recover, as it simply takes too long for axons to reach distal muscles (Robinson, 2000).

Recovery After Peripheral Nerve Injury

Peripheral nerve injuries cause significant disability and morbidity (Asplund et al., 2009). Despite an extensive surgical armamentarium (Millesi et al., 1973; Oberlin et al., 1994; Carlstedt et al., 1995; Giuffre et al., 2010) outcomes of nerve repairs have plateaued offering about 50% functional recovery (Ruijs et al., 2005). Suboptimal recovery of motor functions following axotomy is mainly dependent on three biological phenomena: chronicity of SC denervation, chronicity of neuronal axotomy and misdirection of regenerating axons (Sulaiman and Gordon, 2009). The most important contributor to poor outcome remains the chronic denervation of both neural and terminal SCs (Sulaiman and Gordon, 2000). In humans, a proximal injury along the MN axon can result in months or even a year before the axon terminal reaches NMJs, at which point they will find a chronically denervated environment.

Chronic denervation follows a continuum with worsening effects as duration of denervation lengthens (Fu and Gordon, 1995a; Sulaiman and Gordon, 2000). Only one third of MNs will maintain the ability to regenerate an axon after chronic denervation, increasing the likelihood of

permanent denervation (Fu and Gordon, 1995b; Boyd and Gordon, 2003; Furey et al., 2007).

Finally, regenerating axons grow in a staggered fashion at the injury site resulting in misdirection and delays in regeneration. Misdirection is responsible for up to 30% of axons never reaching the target (Al-Majed et al., 2000).

Motor Regeneration After Prolonged Denervation

Prolonged muscle denervation consistently results in poor recovery and outcome (Höke et al., 2002). Return of innervation to a denervated muscle is function time (Sakuma et al., 2015). Axonal growth rate is constant at 1 to 3 mm/day, therefore longer distances equate longer denervation time. High-grade injuries slow regeneration further prolonging the denervation period. There is a finite window of reinnervation potential after which outcomes are dismal: approximately 10 to 12 months in humans (Campbell, 2008), closer to 12 weeks in the rat (Fu and Gordon, 1995a) and 4 to 6 weeks in the mouse (Ma et al., 2011). These studies indicate that the major contributor to functional recovery is time. Shortening the time of denervation could improve motor recovery. One option to decrease the denervation time would be to shorten the distance between MNs and the targets. This could be accomplished by transplanting MNs in proximity to the muscle.

Cell Replacement Therapy

As discussed previously, MNDs lead to irreversible loss of neurons and are associated with deterioration in physical and cognitive functions. Available treatments are symptomatic. Endogenous neuronal repopulation is not yet possible (Mothe et al., 2011). As such, an alternative source of cells is an attractive possibility to replace diseased neurons.

While the functional outcomes of SCI and MND are similar, their pathophysiology differs. Cell loss in SCI is biphasic. The mechanical disruption injures local neurons but pale in comparison

to the loss of neurons and glia caused by the secondary injury occurring over the following days (Tator and Fehlings, 1991). Half of glia, including oligodendrocytes and astrocytes, perish within a day (Grossman et al., 2001), but their numbers can return to normal after several weeks (Rosenberg et al., 2005). Neuronal loss from either the primary or secondary injury is on the other hand irreversible. Fifteen percent of people suffer complete muscle denervation resulting from MN death at the epicentre of the spinal cord trauma (Thomas and Zijdwind, 2006).

Strategies to replace lost neurons are also attractive in the setting of SCI.

Traumatic peripheral nerve injuries pose a third problem: the discontinuity along the peripheral nervous system between the spinal cord and the muscles prevents the return of innervation either permanently or temporarily long enough for the muscle to be unable to receive innervation. Return of innervation in TPNI is dependent on the duration of denervation, which is associated with the severity of the injury, and the distance axons must travel. Cell replacement therapy could shorten the delay before return of innervation by transplanting MNs in proximity to the muscle.

Sources For Generating Neurons

Primary Cells

Neural transplantation research has come a long way from the initial reports of fetal tissue grafting in Parkinson's disease (Brundin et al., 1986; Dunnett, 1991) and the early clinical trials in Parkinson's (Lindvall et al., 1990) and Huntington's disease (Bachoud-Lévi et al., 2000; Reuter et al., 2008). The double-blinded controlled trials in Parkinson's disease patients that followed were negative (Freed et al., 2001; Olanow et al., 2003), identified important design flaws (Barker et al., 2013a). The presence of contaminating neurons among the dopaminergic grafts that led to dyskinetic effects raised concerns about cell population purity on the outcomes

transplantation (Politis et al., 2010). Ethical and safety concerns led primary cells to fall out of favor for neuronal replacement therapy.

Primary Neural Stem Cells

Primary neural stem and progenitor cells (NSPC) are adult multipotent cells committed to a neuroepithelial lineage and can readily be expanded *in vitro*. NSPCs were first isolated from the adult mouse brain subventricular zone (Reynolds and Weiss, 1992) and later from other regions (Gage and Temple, 2013) including the adult spinal cord peri-central canal region (Weiss et al., 1996; Martens et al., 2002; Kulbatski et al., 2007; Mothe et al., 2011). Spinal cord derived NSPCs transplanted in the injured spinal cord led to improved recovery in mice (Sabelström et al., 2014), likely supporting neuronal survival by the production of several neurotrophic factors (Sabelström et al., 2013). Despite successful transplantation and motor recovery in different models, translation to humans was hindered by the restricted differentiation potential of NSPCs. NSPCs mostly differentiate in glial cells and the diversity neuronal subtypes that can be generated is limited (Steinbeck and Studer, 2015). The proliferative and differentiation properties of NSPCs are dependent on the location and developmental stage of the harvested cells, leading to variable outcomes between progenitor cell lines (Mothe and Tator, 2013). Furthermore, NSPC lines progressively transform to gliogenic only progenitors as passage numbers increase (Naka-Kaneda et al., 2014; Patterson et al., 2014).

Pluripotent Cells

Pluripotent cells can be classified based on their origins into either embryonic stem (ES) cells, harvested from blastocysts, and induced pluripotent stem (iPS) cells, generated from transformed adult somatic cells. Pluripotency requires the ability to differentiate into all three lineages of the embryonic germ layers: ectoderm, mesoderm and endoderm. Pluripotent cells

owe their discovery to studies of embryonal carcinoma cell lines that demonstrated the capacity for self-renewal and for generating mature cell types of all embryonal layers (Kleinsmith and Pierce, 1964). Thirty years of technological advancement was required before cells from the inner cell mass of the mouse blastocyst were isolated, maintained *in vitro*, and used to generate an entire mouse (Nagy et al., 1993). Application of this technology to humans generated the first human ES cell line (Thomson et al., 1998), and has now been applied to multiple ES cell lines. It is now possible to grow ES cells in defined conditions maintaining ES cells undifferentiated in absence of genomic, mitochondrial and epigenetic modifications (Zeng and Rao, 2007; Nistor et al., 2011).

Induced pluripotent stem cells require reprogramming of somatic cells by a combination of transcription factors (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Transformed cells are grown as per ES cell protocols before proliferating colonies are isolated as a new iPS cell line. Hence formed iPS cells were capable of forming teratomas, a defining characteristic of pluripotency. Tumorigenesis is a legitimate risk for transplantation of tissue derived from pluripotent stem cells as they are highly proliferative, unrestricted in their development and highly sensitive to environmental cues (Matsuda et al., 2009; Wyatt and Keirstead, 2012).

Generation Of Motoneurons

Motoneuron Embryogenesis

During embryogenesis, the inner cell mass derived from the epiblast differentiates into the ectoderm and endoderm. Ectodermal tissue further differentiates into neuroectoderm from which neuroprogenitor cells are derived (Wilson and Edlund, 2001). In order to generate spinal MNs, neuroprogenitors must be caudalized with retinoid acid (RA; Wagner et al., 1992; Maden, 2007) and ventralized with Sonic Hedgehog (Shh; Echelard et al., 1993; Jessell, 2000). Under the

differentiating effect of Shh, five neuroprogenitor classes are generated in a ventral to dorsal pattern: V3, Vm, V2, V1 and V0. Sox1⁺ neuroprogenitors produce Olig2⁺ MN progenitors, sequentially expressing Isl1, Lhx3 and finally Hb9, a marker of post-mitotic MN (Arber et al., 1999; Thaler et al., 1999).

MNs appear three to five days after the addition of patterning factors *in vitro* (Wichterle et al., 2002; Miles et al., 2004). Continued exposure to RA establishes a MN identity typically of high cervical spinal cord (Jessell, 2000; Maden, 2007; Peljto et al., 2010).

Generation Of Motoneurons From Pluripotent Cells

Pluripotent stem cells spontaneously differentiate upon removal of differentiation inhibitory factors (Evans, 2011). Spontaneous differentiation via the formation of embryonic bodies, cellular aggregates formed in non-adherent culture conditions (Odorico et al., 2001), leads to a poor efficiency in generating neurons and a large contamination by non-neural tissue (Bain et al., 1995).

Adding Shh and RA during the differentiation process skews a significant portion of the progeny, about 30%, towards a motoneuronal path (Wichterle et al., 2002). The remaining neurons generated during this process include: glutamatergic (10%), GABAergic (15%), and glycinergic (6%) neurons. A significant proportion of these neurons belong to the V0 (12%) and V2 (13%) populations. Glia accounts for slightly less than 30%: astrocytes (21%) and oligodendrocytes (7%; Deshpande et al., 2006).

Embryonic Stem Cell-Derived Motoneurons

Motoneurons derived from embryonic stem cells progressively acquire passive and active membrane properties over a four-day period following complete differentiation. By that time, their passive membrane properties are equivalent to E18 phrenic MNs (Miles et al., 2004).

Motoneurons also express GABA, glycine, and glutamate receptors, consistent with *in vivo* MNs of similar embryonic age (Wu et al., 1992; Gao and Ziskind-Conhaim, 1995; Miles et al., 2004; Stein et al., 2004). Like endogenous MNs, ESCMNs fire repetitive action potentials in response to current injection and demonstrate spike-frequency adaptation and rebound action potentials. These firing characteristics are compatible with developing MNs (Martin-Caraballo and Greer, 1999). Finally, ESCMNs lack apamin-sensitive after-hyperpolarization potentials (AHPs; Miles et al., 2004). Apamin-sensitive AHPs originate from clusters of SK channels at C-bouton synapses (Deardorff et al., 2012) but form only in the post-natal period (Thana, Panek and Brownstone, personal communication). The lack of an apamin-sensitive AHP in ESCMNs suggests a young embryonic age.

The ultimate characteristic of MNs is their ability to form functional NMJs with muscle fibres. As such, ESCMNs form NMJs when cultured with myotubes (Miles et al., 2004). When transplanted into the developing chick spinal cord, ESCMNs integrate into the spinal network (Soundararajan et al., 2006) and demonstrate an axonal targeting identity consistent with medial motor column MNs (Soundararajan et al., 2006; 2007; 2010). When transplanted in the adult mouse tibial nerve, ESCMNs innervate muscle fibres and restore contraction upon stimulation (Yohn et al., 2008).

iPS Cell-Derived Motor Neurons

Social and technological challenges raise questions towards the use of embryonic tissue especially with transplantation of human cells derived from embryonic stem cells. It would also be attractive to be able to avoid immunosuppression required to prevent rejection of allogenic transplanted tissue. Induced pluripotent stem cells serve this purpose. This technology opened the venue for generation of human pluripotent cells from adult fibroblasts (Takahashi et al., 2007; Park et al., 2008), allowing the generation of patient-specific stem cells. MNs were

subsequently generated from human iPS cells derived from ALS patients (Dimos et al., 2008). MNs generated from iPS cells reproduced the electrical, axonal guidance and synaptogenesis found in ESCMNs (Toma et al., 2015).

Direct Conversion Of Fibroblasts To Motoneurons

The reprogramming of fibroblasts to MNs initially required the establishment of a pluripotent state from which MN differentiation sequence could be initiated. Reprogramming fibroblasts directly into MNs could prevent spurious unwanted outcomes seen with pluripotent cells. Three lineage-specific transcription factors (Brn2 / Pou3f2, Ascl1, and Myt11) directly converted fibroblasts into neurons (Vierbuchen et al., 2010) and seven factors (Ascl1, Brn2, Myt11, Lhx3, Hb9, Isl1, and Ngn2) generated MNs (Son et al., 2011). These induced MNs demonstrated electrophysiological properties of endogenous MNs similar to those found in ES and iPS cell-derived MNs.

Restoration Of Motor Functions

Functional Electrical Stimulation

Functional electrical stimulation (FES) is the restoration of function through electrical stimulation of the central or peripheral nervous system (Ho et al., 2014). FES have been widely used in rehabilitation and neurosurgical centers to restore hearing, bladder control, muscle contraction, respiration, and to control pain, spasticity, tremor and rigidity (Prochazka et al., 2001).

Early application of FES reverses the changes associated with skeletal muscle denervation including: fibrillation potentials, acetylcholine hypersensitivity, expression of TTX-resistant sodium channels, and loss of muscle mass (Cisterna et al., 2014). Muscle atrophy can be prevented and sometimes reversed by exogenous electrical stimulation (Salmons et al., 2005).

Brief periods of stimulation can prevent atrophy (Baldi et al., 1998), whereas prolonged periods increase muscle volume, strength and endurance (Shields and Dudley-Javoroski, 2007).

FES requires an intact neuromuscular system to work properly (Popovic et al., 1991). In the absence of motor innervation, as seen in TPNIs, MNDs and some cases of SCI, FES is not efficient currents required to activation muscle leads to side effects (Ho et al., 2014).

Transplantation of neuron could provide the missing motor innervation for FES to work.

Motoneuron Transplantation

Embryonic MNs transplanted into the spinal cord can survive and innervate muscle (Sieradzan and Vrbová, 1989). Since axons from transplanted MNs need to growth from the spinal to the muscle, prolonged denervation remains problematic. Transplantation of neurons into the peripheral nervous system was first demonstrated using cortical neurons (Bernstein and Tang, 1984), and was extended by Bunge, Thomas and colleagues using embryonic rat ventral spinal cord cells (Erb et al., 1993; Thomas et al., 2000; 2003). More recently, we and others have used pluripotent cells to generate MNs (Harper et al., 2004; Deshpande et al., 2006; Yohn et al., 2008; Toma et al., 2015). Transplanting MN into peripheral nerves in proximity to the affected muscle(s) could decrease the denervation time and potentially improve motor recovery.

Direct Activation Of Muscle Fibres

Direct electrical stimulation of muscle fibres is possible, but in the absence of innervation is inefficient and requires currents large enough to induce tissue damage, electrode corrosion, stimulation spread and activation of pain receptors (Popovic et al., 1991; Ragnarsson, 2008).

Channelrhodopsin-expressing neurons transplanted in the tibial nerve can restore muscular control using light emitted by a nerve cuff (Bryson et al., 2014). Yet, this technology relies on a functional neuromuscular system. Bypassing the neural system all together by directly

depolarizing muscle fibres can be achieved with optogenetics and might provide control over muscle to restore function.

Optogenetics take advantage of the microbial opsin channelrhodopsin-2 (ChR2) that upon light activation depolarizes neuronal membranes (Nagel et al., 2003; Boyden et al., 2005). ChR2 is a nonselective channel conducting sodium, potassium, proton and calcium ions. ChR2 is maximally excited at 470 nm, has a fast on-rate of less than 1.5 ms and a closing rate of 10 to 13 ms. High level of desensitization can lead to inconsistencies in responses more prominent with repetitive stimulation or continuous light pulses (Ishizuka et al., 2006; Lin et al., 2009). The first published variant of ChR2 was ChR2-H134R developed to decreased desensitization and improves expression in mammalian tissue (Nagel et al., 2005). ChR2-H134R is a well-characterized channel demonstrating reliable expression and kinetic properties compatible with muscle excitation contraction coupling.

Light-emitting diodes (LED) are a practical source for experiments with light stimulation for multiple reasons. LEDs are inexpensive, have a long lifetime, demonstrate rapid shuttering and intensity control via controlled current input, can be coupled to sub-millimeter range optical fibres for distant transmission, and are small enough to be implantable or mounted on a rodent (Huber et al., 2008; Lin, 2012).

Research Chapters

Chapter Two: Preventing Tumorigenesis Improves Innervation By Transplanted Embryonic Stem Cell Derived Motoneurons

To restore motor function after denervation from MNDs, SCIs and TPNIs, we transplanted ESCMNs into the tibial nerve to assess innervation after prolonged denervation. We focused on ES cells and MNs derived from ES cells has both had been well characterized and were

consistent in their differentiation and maturation. We show a progressive decline in reinnervation as denervation period lengthened, and growth of tumours in half of transplants. Despite neuronal differentiation, residual pluripotent cells remains and generated tumours. Ablation of pluripotent cells post differentiation led to survival and functional muscle innervation by transplanted ESCMNs.

Chapter Three: Direct Optical Activation Of Skeletal Muscle Fibres Efficiently Controls Muscle Contraction And Attenuates Denervation Atrophy

In the absence of innervating motor axons, neural prostheses fail to restore motor function to paralyzed muscles. MNDs, SCIs and TPNIs are examples where muscles are partially or completely devoid of innervation by MNs. An alternative to electrical stimulation for neural prostheses, we aimed at depolarizing muscle fibres in a controllable fashion in spite of denervation. We show that expression of ChR2-H134R along the sarcolemma and T-tubules allow optical control of skeletal muscle contraction with comparable forces to electrical stimulation. Contraction could also be adjusted by light pulse intensity, duration and frequency. This chapter shows that ChR can be used to restore motor function to permanently denervated muscles.

Chapter Four: Spontaneous Circuit Activity Of Transplanted Embryonic Stem Cell-Derived Motoneurons

Therapeutic transplantation of neurons derived from stem cells is an attractive therapy in the treatment of diseases leading to denervation where self-regeneration is not a possibility. We transplanted ESCMNs in the transected tibial nerve to innervate the medial gastrocnemius post denervation. Half of successful transplants developed an enlargement along the nerve at the site of transplantation and autonomously generated muscle contractions with high variability activity between transplants. The activity arrested by blocking glutamate transmission and was

modulated by GABA/glycine and muscarinic inhibitors. The formation of an active circuitry among transplanted ESCMNs emphasizes the intrinsic nature of neurons to form synaptic network. These findings accentuate the necessity to better understand the maturation and synaptogenesis of stem cell-derived neurons post-transplantation.

References

- Ahn H, Bailey CS, Rivers CS, Noonan VK, Tsai EC, Fournery DR, Attabib N, Kwon BK, Christie SD, Fehlings MG, Finkelstein J, Hurlbert RJ, Townson A, Parent S, Drew B, Chen J, Dvorak MF, Rick Hansen Spinal Cord Injury Registry Network (2015) Effect of older age on treatment decisions and outcomes among patients with traumatic spinal cord injury. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne* 187:873–880.
- Al-Majed AA, Neumann CM, Brushart TM, Gordon T (2000) Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. *J Neurosci* 20:2602–2608.
- Arber S, Han B, Mendelsohn M, Smith M, Jessell TM, Sockanathan S (1999) Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. *Neuron* 23:659–674.
- Asplund M, Nilsson M, Jacobsson A, Holst von H (2009) Incidence of Traumatic Peripheral Nerve Injuries and Amputations in Sweden between 1998 and 2006. *Neuroepidemiology* 32:217–228.
- Bachoud-Lévi AC et al. (2000) Motor and cognitive improvements in patients with Huntington's disease after neural transplantation. *The Lancet* 356:1975–1979.
- Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI (1995) Embryonic stem cells express neuronal properties in vitro. *Dev Biol* 168:342–357.
- Baldi JC, Jackson RD, Moraille R, Mysiw WJ (1998) Muscle atrophy is prevented in patients with acute spinal cord injury using functional electrical stimulation. *Spinal Cord* 36:463–469.
- Barohn RJ, Amato AA (2013) Pattern-recognition approach to neuropathy and neuronopathy. *Neurologic clinics* 31:343–361.
- Bellingham MC (2011) A review of the neural mechanisms of action and clinical efficiency of riluzole in treating amyotrophic lateral sclerosis: what have we learned in the last decade? *CNS Neurosci Ther* 17:4–31.
- Bernstein JJ, Tang Y (1984) Structure and function of fetal cortex implanted into degenerating peripheral nerve of adult rat. *Brain Res* 324:243–251.
- Boyd JG, Gordon T (2003) Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons in vivo. *Exp Neurol* 183:610–619.
- Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (2005) Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci* 8:1263–1268.
- Brundin P, Nilsson OG, Strecker RE, Lindvall O, Astedt B, Björklund A (1986) Behavioural effects of human fetal dopamine neurons grafted in a rat model of Parkinson's disease. *Experimental brain research Experimentelle Hirnforschung Expérimentation cérébrale* 65:235–240.

- Bryson JB, Machado CB, Crossley M, Stevenson D, Bros-Facer V, Burrone J, Greensmith L, Lieberam I (2014) Optical Control of Muscle Function by Transplantation of Stem Cell-Derived Motor Neurons in Mice. *Science* 344:94–97.
- Buchthal F, Kühl V (1979) Nerve conduction, tactile sensibility, and the electromyogram after suture or compression of peripheral nerve: a longitudinal study in man. *J Neurol Neurosurg Psychiatr* 42:436–451.
- Cajal SY (1959) Degeneration & regeneration of the nervous system.
- Campbell WW (2008) Evaluation and management of peripheral nerve injury. *Clinical Neurophysiology* 119:1951–1965.
- Carlstedt T, Grane P, Hallin RG, Norén G (1995) Return of function after spinal cord implantation of avulsed spinal nerve roots. *The Lancet* 346:1323–1325.
- Chancellor AM, Slattery JM, Fraser H, Swingler RJ, Holloway SM, Warlow CP (1993) The prognosis of adult-onset motor neuron disease: a prospective study based on the Scottish Motor Neuron Disease Register. *Journal of Neurology* 240:339–346.
- Cisterna BA, Cardozo C, Sáez JC (2014) Neuronal involvement in muscular atrophy. *Front Cell Neurosci* 8:405.
- de Carvalho M, Dengler R, Eisen A, England JD, Kaji R, Kimura J, Mills K, Mitsumoto H, Nodera H, Shefner J, Swash M (2008) Electrodiagnostic criteria for diagnosis of ALS. *Clinical Neurophysiology* 119:497–503.
- Deardorff AS, Romer SH, Deng Z, Bullinger KL, Nardelli P, Cope TC, Fyffe REW (2012) Expression of postsynaptic Ca²⁺-activated K⁺ (SK) channels at C-bouton synapses in mammalian lumbar-motoneurons. *J Physiol (Lond)*.
- DeJesus-Hernandez M et al. (2011) Expanded GGGGCC Hexanucleotide Repeat in Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS. *Neuron* 72:245–256.
- del Aguila MA, Longstreth WT, McGuire V, Koepsell TD, van Belle G (2003) Prognosis in amyotrophic lateral sclerosis: A population-based study. *Neurology* 60:813–819.
- Deshpande DM, Kim Y-S, Martinez T, Carmen J, Dike S, Shats I, Rubin LL, Drummond J, Krishnan C, Hoke A, Maragakis N, Shefner J, Rothstein JD, Kerr DA (2006) Recovery from paralysis in adult rats using embryonic stem cells. *Ann Neurol* 60:32–44.
- Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Eggan K (2008) Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons. *Science* 321:1218–1221.
- Dunnett SB (1991) Towards a neural transplantation therapy for Parkinson's disease: experimental principles from animal studies. *Acta Neurochir Suppl (Wien)* 52:35–38.

- Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75:1417–1430.
- Erb DE, Mora RJ, Bunge RP (1993) Reinnervation of adult rat gastrocnemius muscle by embryonic motoneurons transplanted into the axotomized tibial nerve. *Exp Neurol* 124:372–376.
- Evans M (2011) Discovering pluripotency: 30 years of mouse embryonic stem cells. *Nat Rev Mol Cell Biol* 12:680–686.
- Feldman EL, Boulis NM, Hur J, Johe K, Rutkove SB, Federici T, Polak M, Bordeau J, Sakowski SA, Glass JD (2014) Intraspinal neural stem cell transplantation in amyotrophic lateral sclerosis: Phase 1 trial outcomes. *Ann Neurol* 75:363–373.
- Fowler TJ, Danta G, Gilliatt RW (1972) Recovery of nerve conduction after a pneumatic tourniquet: observations on the hind-limb of the baboon. *J Neurol Neurosurg Psychiatr* 35:638–647.
- Fu SY, Gordon T (1995a) Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. *J Neurosci* 15:3886–3895.
- Fu SY, Gordon T (1995b) Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy. *J Neurosci* 15:3876–3885.
- Furey MJ, Midha R, Xu Q-G, Belkas J, Gordon T (2007) Prolonged Target Deprivation Reduces the Capacity of Injured Motoneurons to Regenerate. *60:723??733*.
- Furlan JC, Noonan V, Singh A, Fehlings MG (2011) Assessment of impairment in patients with acute traumatic spinal cord injury: a systematic review of the literature. *J Neurotrauma* 28:1445–1477.
- Gage FH, Temple S (2013) Neural Stem Cells: Generating and Regenerating the Brain. *Neuron* 80:588–601.
- Gan LS, Prochazka A, Bornes TD, Denington AA, Chan KM (2007) A new means of transcutaneous coupling for neural prostheses. *IEEE transactions on bio-medical engineering* 54:509–517.
- Gao BX, Ziskind-Conhaim L (1995) Development of glycine- and GABA-gated currents in rat spinal motoneurons. *J Neurophysiol* 74:113–121.
- Giuffre JL, Kakar S, Bishop AT, Spinner RJ, Shin AY (2010) Current Concepts of the Treatment of Adult Brachial Plexus Injuries. *J Hand Surg Am* 35:678–688.
- Grossman SD, Rosenberg LJ, Wrathall JR (2001) Temporal-spatial pattern of acute neuronal and glial loss after spinal cord contusion. *Exp Neurol* 168:273–282.

- Harper JM, Krishnan C, Darman JS, Deshpande DM, Peck S, Shats I, Backovic S, Rothstein JD, Kerr DA (2004) Axonal growth of embryonic stem cell-derived motoneurons in vitro and in motoneuron-injured adult rats. *Proc Natl Acad Sci USA* 101:7123–7128.
- Hegedus J, Putman CT, Tyreman N, Gordon T (2008) Preferential motor unit loss in the SOD1 G93A transgenic mouse model of amyotrophic lateral sclerosis. *J Physiol (Lond)* 586:3337–3351.
- Ho CH, Triolo RJ, Elias AL, Kilgore KL, DiMarco AF, Bogie K, Vette AH, Audu ML, Kobetic R, Chang SR, Chan KM, Dukelow S, Bourbeau DJ, Brose SW, Gustafson KJ, Kiss ZHT, Mushahwar VK (2014) Functional electrical stimulation and spinal cord injury. *Phys Med Rehabil Clin N Am* 25:631–54–ix.
- Hoffman H (1950) Local re-innervation in partially denervated muscle; a histophysiological study. *Aust J Exp Biol Med Sci* 28:383–397.
- Höke A, Gordon T, Zochodne DW, Sulaiman OAR (2002) A decline in glial cell-line-derived neurotrophic factor expression is associated with impaired regeneration after long-term Schwann cell denervation. *Exp Neurol* 173:77–85.
- Huber D, Petreanu L, Ghitani N, Ranade S, Hromádka T, Mainen Z, Svoboda K (2008) Sparse optical microstimulation in barrel cortex drives learned behaviour in freely moving mice. *Nature* 451:61–64.
- Ishizuka T, Kakuda M, Araki R, Yawo H (2006) Kinetic evaluation of photosensitivity in genetically engineered neurons expressing green algae light-gated channels. *Neuroscience Research* 54:85–94.
- Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nature Reviews Genetics* 1:20–29.
- Kang H, Tian L, Mikesh M, Lichtman JW, Thompson WJ (2014) Terminal Schwann cells participate in neuromuscular synapse remodeling during reinnervation following nerve injury. *Journal of Neuroscience* 34:6323–6333.
- Kleinsmith LJ, Pierce GB (1964) Multipotentiality of single embryonal carcinoma cells. *Cancer Res* 24:1544–1551.
- Knutsdottir S, Thorisdottir H, Sigvaldason K, Jónsson H, Björnsson A, Ingvarsson P (2012) Epidemiology of traumatic spinal cord injuries in Iceland from 1975 to 2009. *Spinal Cord* 50:123–126.
- Krueger H, Noonan VK, Trenaman LM, Joshi P, Rivers CS (2013) The economic burden of traumatic spinal cord injury in Canada. *Chronic Dis Inj Can* 33:113–122.
- Kulbatski I, Mothe AJ, Keating A, Hakamata Y, Kobayashi E, Tator CH (2007) Oligodendrocytes and radial glia derived from adult rat spinal cord progenitors: morphological and immunocytochemical characterization. *J Histochem Cytochem* 55:209–222.

- Li H, Terenghi G, Hall SM (1997) Effects of delayed re-innervation on the expression of c-erbB receptors by chronically denervated rat Schwann cells in vivo. *Glia* 20:333–347.
- Lin JY (2012) Optogenetic excitation of neurons with channelrhodopsins: light instrumentation, expression systems, and channelrhodopsin variants. *Prog Brain Res* 196:29–47.
- Lin JY, Lin MZ, Steinbach P, Tsien RY (2009) Characterization of engineered channelrhodopsin variants with improved properties and kinetics. *Biophysical Journal* 96:1803–1814.
- Lindvall O, Brundin P, Widner H, Rehnström S, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, Marsden CD (1990) Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science* 247:574–577.
- Ma CHE, Omura T, Cobos EJ, Latremoliere A, Ghasemlou N, Brenner GJ, van Veen E, Barrett L, Sawada T, Gao F, Coppola G, Gertler F, Costigan M, Geschwind D, Woolf CJ (2011) Accelerating axonal growth promotes motor recovery after peripheral nerve injury in mice. *J Clin Invest* 121:4332–4347.
- Maden M (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nat Rev Neurosci* 8:755–765.
- Martens DJ, Seaberg RM, van der Kooy D (2002) In vivo infusions of exogenous growth factors into the fourth ventricle of the adult mouse brain increase the proliferation of neural progenitors around the fourth ventricle and the central canal of the spinal cord. *Eur J Neurosci* 16:1045–1057.
- Martin-Caraballo M, Greer JJ (1999) Electrophysiological properties of rat phrenic motoneurons during perinatal development. *J Neurophysiol* 81:1365–1378.
- Matsuda R, Yoshikawa M, Kimura H, Ojui Y, Nakase H, Nishimura F, Nonaka J-I, Toriumi H, Yamada S, Nishiofuku M, Moriya K, Ishizaka S, Nakamura M, Sakaki T (2009) Cotransplantation of mouse embryonic stem cells and bone marrow stromal cells following spinal cord injury suppresses tumor development. *Cell transplantation* 18:39–54.
- Miles GB, Yohn DC, Wichterle H, Jessell TM, Rafuse VF, Brownstone RM (2004) Functional properties of motoneurons derived from mouse embryonic stem cells. *Journal of Neuroscience* 24:7848–7858.
- Miller RG, Mitchell JD, Moore DH (2012) Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). Miller RG, ed. *Cochrane Database Syst Rev* 3:CD001447.
- Miller TM et al. (2013) An antisense oligonucleotide against SOD1 delivered intrathecally for patients with SOD1 familial amyotrophic lateral sclerosis: a phase 1, randomised, first-in-man study. *The Lancet Neurology* 12:435–442.
- Millesi H, Meissl G, Berger A (1973) The interfascicular nerve-grafting of median and ulnar nerves. *Plast Reconstr Surg* 51:233.
- Milner Brown HS, Stein RB (1975) The relation between the surface electromyogram and muscular force. *J Physiol (Lond)* 246:549–569.

- Moritani T, deVries HA (1979) Neural factors versus hypertrophy in the time course of muscle strength gain. *Am J Phys Med* 58:115–130.
- Mothe AJ, Tator CH (2013) Review of transplantation of neural stem/progenitor cells for spinal cord injury. *Int J Dev Neurosci* 31:701–713.
- Mothe AJ, Zahir T, Santaguida C, Cook D, Tator CH (2011) Neural stem/progenitor cells from the adult human spinal cord are multipotent and self-renewing and differentiate after transplantation. *PLoS ONE* 6:e27079.
- Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A (2005) Light Activation of Channelrhodopsin-2 in Excitable Cells of *Caenorhabditis elegans* Triggers Rapid Behavioral Responses. *Current Biology* 15:2279–2284.
- Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, Bamberg E (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc Natl Acad Sci USA* 100:13940–13945.
- Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA* 90:8424–8428.
- Naka-Kaneda H, Nakamura S, Igarashi M, Aoi H, Kanki H, Tsuyama J, Tsutsumi S, Aburatani H, Shimazaki T, Okano H (2014) The miR-17/106-p38 axis is a key regulator of the neurogenic-to-gliogenic transition in developing neural stem/progenitor cells. *Proc Natl Acad Sci USA* 111:1604–1609.
- Navarro X, Vivó M, Valero-Cabré A (2007) Neural plasticity after peripheral nerve injury and regeneration. *Prog Neurobiol* 82:163–201.
- Nistor G, Siegenthaler MM, Poirier SN, Rossi S, Poole AJ, Charlton ME, McNeish JD, Airriess CN, Keirstead HS (2011) Derivation of high purity neuronal progenitors from human embryonic stem cells. Cooney AJ, ed. *PLoS ONE* 6:e20692.
- Noble J, Munro CA, Prasad VSSV, Midha R (1998) Analysis of Upper and Lower Extremity Peripheral Nerve Injuries in a Population of Patients with Multiple Injuries. *The Journal of Trauma: Injury, Infection, and Critical Care* 45:116–122.
- Noonan VK, Fingas M, Farry A, Baxter D, Singh A, Fehlings MG, Dvorak MF (2012) Incidence and Prevalence of Spinal Cord Injury in Canada: A National Perspective. *Neuroepidemiology* 38:219–226.
- Oberlin C, Béal D, Leechavengvongs S, Salon A, Dauge MC, Sarcy JJ (1994) Nerve transfer to biceps muscle using a part of ulnar nerve for C5–C6 avulsion of the brachial plexus: Anatomical study and report of four cases. *J Hand Surg Am* 19:232–237.
- Odorico JS, Kaufman DS, Thomson JA (2001) Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* 19:193–204.

- Park I-H, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ (2008) Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451:141–146.
- Patterson M, Gaeta X, Loo K, Edwards M, Smale S, Cinkornpumin J, Xie Y, Listgarten J, Azghadi S, Douglass SM, Pellegrini M, Lowry WE (2014) let-7 miRNAs can act through notch to regulate human gliogenesis. *Stem Cell Reports* 3:758–773.
- Peljto M, Dasen JS, Mazzoni EO, Jessell TM, Wichterle H (2010) Functional diversity of ESC-derived motor neuron subtypes revealed through intraspinal transplantation. *Cell Stem Cell* 7:355–366.
- Pirouzmand F (2010) Epidemiological trends of spine and spinal cord injuries in the largest Canadian adult trauma center from 1986 to 2006. *J Neurosurg Spine* 12:131–140.
- Popovic D, Gordon T, Rafuse VF, Prochazka A (1991) Properties of implanted electrodes for functional electrical stimulation. *Ann Biomed Eng* 19:303–316.
- Prochazka A, Mushahwar VK, McCreery DB (2001) Neural prostheses. *J Physiol (Lond)* 533:99–109.
- Ragnarsson KT (2008) Functional electrical stimulation after spinal cord injury: current use, therapeutic effects and future directions. *Spinal Cord* 46:255–274.
- Renton AE et al. (2011) A Hexanucleotide Repeat Expansion in C9ORF72 Is the Cause of Chromosome 9p21-Linked ALS-FTD. *Neuron* 72:257–268.
- Reuter I, Tai YF, Pavese N, Chaudhuri KR, Mason S, Polkey CE, Clough C, Brooks DJ, Barker RA, Piccini P (2008) Long-term clinical and positron emission tomography outcome of fetal striatal transplantation in Huntington's disease. *J Neurol Neurosurg Psychiatr* 79:948–951.
- Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255:1707–1710.
- Reynolds ML, Woolf CJ (1992) Terminal Schwann cells elaborate extensive processes following denervation of the motor endplate. *J Neurocytol* 21:50–66.
- Robinson LR (2000) Traumatic injury to peripheral nerves. *Muscle Nerve* 23:863–873.
- Rosenberg LJ, Zai LJ, Wrathall JR (2005) Chronic alterations in the cellular composition of spinal cord white matter following contusion injury. *Glia* 49:107–120.
- Ruijs ACJ, Jaquet J-B, Kalmijn S, Giele H, Hovius SER (2005) Median and ulnar nerve injuries: a meta-analysis of predictors of motor and sensory recovery after modern microsurgical nerve repair. *Plast Reconstr Surg* 116:484–94–discussion495–6.
- Sabelström H, Stenudd M, Frisén J (2014) Neural stem cells in the adult spinal cord. *Exp Neurol* 260:44–49.

- Sabelström H, Stenudd M, Réu P, Dias DO, Elfineh M, Zdunek S, Damberg P, Göritz C, Frisé J (2013) Resident neural stem cells restrict tissue damage and neuronal loss after spinal cord injury in mice. *Science* 342:637–640.
- Sakuma M, Gorski G, Sheu S-H, Lee S, Barrett LB, Singh B, Omura T, Latremoliere A, Woolf CJ (2015) Lack of motor recovery after prolonged denervation of the neuromuscular junction is not due to regenerative failure Gaspar P, ed. *Eur J Neurosci*:n/a–n/a.
- Salmons S, Ashley Z, Sutherland H, Russold MF, Li F, Jarvis JC (2005) Functional Electrical Stimulation of Denervated Muscles: Basic Issues. *Artificial organs* 29:199–202.
- Seddon H (1975) *Surgical Disorders of the Peripheral Nerves*. Churchill Livingstone.
- Sekhon LH, Fehlings MG (2001) Epidemiology, demographics, and pathophysiology of acute spinal cord injury. *Spine* 26:S2–S12.
- Selecki BR, Ring IT, Simpson DA, Vanderfield GK, Sewell MF (1982) Trauma to the central and peripheral nervous systems part ii: a statistical profile of surgical treatment new south wales 1977. *ANZ J Surg* 52:111–116.
- Shields RK, Dudley-Javoroski S (2007) Musculoskeletal Adaptations in Chronic Spinal Cord Injury: Effects of Long-term Soleus Electrical Stimulation Training. *Neurorehabilitation and neural repair* 21:169–179.
- Sieradzan K, Vrbová G (1989) Replacement of missing motoneurons by embryonic grafts in the rat spinal cord. *NSC* 31:115–130.
- Siironen J, Sandberg M, Vuorinen V, Røyttä M (1992) Expression of type I and III collagens and fibronectin after transection of rat sciatic nerve. Reinnervation compared with denervation. *Lab Invest* 67:80–87.
- Singh A, Tetreault L, Kalsi-Ryan S, Nouri A, Fehlings MG (2014) Global prevalence and incidence of traumatic spinal cord injury. *Clin Epidemiol* 6:309–331.
- Son EY, Ichida JK, Wainger BJ, Toma JS, Rafuse VF, Woolf CJ, Eggan K (2011) Conversion of Mouse and Human Fibroblasts into Functional Spinal Motor Neurons. *Stem Cell* 9:205–218.
- Son YJ, Thompson WJ (1995) Nerve sprouting in muscle is induced and guided by processes extended by Schwann cells. *Neuron* 14:133–141.
- Soundararajan P, Fawcett JP, Rafuse VF (2010) Guidance of postural motoneurons requires MAPK/ERK signaling downstream of fibroblast growth factor receptor 1. *Journal of Neuroscience* 30:6595–6606.
- Soundararajan P, Lindsey BW, Leopold C, Rafuse VF (2007) Easy and rapid differentiation of embryonic stem cells into functional motoneurons using sonic hedgehog-producing cells. *Stem Cells* 25:1697–1706.

- Soundararajan P, Miles GB, Rubin LL, Brownstone RM, Rafuse VF (2006) Motoneurons derived from embryonic stem cells express transcription factors and develop phenotypes characteristic of medial motor column neurons. *Journal of Neuroscience* 26:3256–3268.
- Statland JM, Barohn RJ, McVey AL, Katz JS, Dimachkie MM (2015) Patterns of Weakness, Classification of Motor Neuron Disease, and Clinical Diagnosis of Sporadic Amyotrophic Lateral Sclerosis. *Neurologic clinics* 33:735–748.
- Stein V, Hermans-Borgmeyer I, Jentsch TJ, Hübner CA (2004) Expression of the KCl cotransporter KCC2 parallels neuronal maturation and the emergence of low intracellular chloride. *J Comp Neurol* 468:57–64.
- Steinbeck JA, Studer L (2015) Moving Stem Cells to the Clinic: Potential and Limitations for Brain Repair. *Neuron* 86:187–206.
- Sulaiman OA, Gordon T (2000) Effects of short- and long-term Schwann cell denervation on peripheral nerve regeneration, myelination, and size. *Glia* 32:234–246.
- Sulaiman OAR, Gordon T (2009) Role of chronic Schwann cell denervation in poor functional recovery after nerve injuries and experimental strategies to combat it. 65:105–114.
- Sunderland S (1978) *Nerves And Nerve Injuries*. Churchill Livingstone.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872.
- Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
- Tator CH, Fehlings MG (1991) Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. *J Neurosurg* 75:15–26.
- Tetzlaff W, Okon EB, Karimi-Abdolrezaee S, Hill CE, Sparling JS, Plemel JR, Plunet WT, Tsai EC, Baptiste D, Smithson LJ, Kawaja MD, Fehlings MG, Kwon BK (2011) A Systematic Review of Cellular Transplantation Therapies for Spinal Cord Injury. <http://dxdoiorg/101089/neu20091177> 28:1611–1682.
- Thaler J, Harrison K, Sharma K, Lettieri K, Kehrl J, Pfaff SL (1999) Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. *Neuron* 23:675–687.
- Thomas CK, Erb DE, Grumbles RM, Bunge RP (2000) Embryonic cord transplants in peripheral nerve restore skeletal muscle function. *J Neurophysiol* 84:591–595.
- Thomas CK, Sesodia S, Erb DE, Grumbles RM (2003) Properties of medial gastrocnemius motor units and muscle fibers reinnervated by embryonic ventral spinal cord cells. *Exp Neurol* 180:25–31.

- Thomas CK, Zijdewind I (2006) Fatigue of muscles weakened by death of motoneurons. *Muscle Nerve* 33:21–41.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147.
- Toma JS, Shettar BC, Chipman PH, Pinto DM, Borowska JP, Ichida JK, Fawcett JP, Zhang Y, Eggan K, Rafuse VF (2015) Motoneurons derived from induced pluripotent stem cells develop mature phenotypes typical of endogenous spinal motoneurons. *Journal of Neuroscience* 35:1291–1306.
- Traxinger K, Kelly C, Johnson BA, Lyles RH, Glass JD (2013) Prognosis and epidemiology of amyotrophic lateral sclerosis: Analysis of a clinic population, 1997-2011. *Neurol Clin Pract* 3:313–320.
- Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Südhof TC, Wernig M (2010) Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463:1035–1041.
- Wagner M, Han B, Jessell TM (1992) Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. *Development* 116:55–66.
- Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA (1996) Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci* 16:7599–7609.
- Wichterle H, Lieberam I, Porter JA, Jessell TM (2002) Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110:385–397.
- Wilson SI, Edlund T (2001) Neural induction: toward a unifying mechanism. *Nat Neurosci* 4 Suppl:1161–1168.
- Wu WL, Ziskind-Conhaim L, Sweet MA (1992) Early development of glycine- and GABA-mediated synapses in rat spinal cord. *J Neurosci* 12:3935–3945.
- Wyatt LA, Keirstead HS (2012) Stem cell-based treatments for spinal cord injury. *Prog Brain Res* 201:233–252.
- Yohn DC, Miles GB, Rafuse VF, Brownstone RM (2008) Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy. *Journal of Neuroscience* 28:12409–12418.
- You S, Petrov T, Chung PH, Gordon T (1997) The expression of the low affinity nerve growth factor receptor in long-term denervated Schwann cells. *Glia* 20:87–100.
- Zeng X, Rao MS (2007) Human embryonic stem cells: long term stability, absence of senescence and a potential cell source for neural replacement. *NSC* 145:1348–1358.

Chapter Two: Preventing Tumorigenesis Improves Innervation By Transplanted Embryonic Stem Cell Derived Motoneurons

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Abstract

Nerve injuries resulting in prolonged periods of denervation often result in poor recovery of motor function. We have previously shown that embryonic stem cell-derived motoneurons transplanted at the time of transection into a peripheral nerve can functionally reinnervate muscle. For clinical relevance, we now focused on delaying transplantation to assess reinnervation after prolonged denervation.

Embryonic stem derived motoneurons were transplanted into the distal segments of transected tibial nerves in adult mice after prolonged denervation of one to eight weeks. Twitch and tetanic forces were measured *ex vivo* three months post transplantation. Tissue was harvested from the transplants for culture and immunohistochemical analysis.

We show that success of reinnervation decreased as denervation period lengthened, and failed altogether in transplants denervated for eight weeks. Furthermore, teratocarcinomas developed in about one-half of transplants. A residual multipotent cell population (~6% of cells) was found despite neural differentiation. Exposure to the antimetabolic drug mitomycin C eliminated this multipotent population *in vitro* while preserving motoneurons. Treating differentiated embryoid bodies prior to delayed transplantation prevented tumour formation and resulted in twitch and tetanic forces similar to those in animals transplanted acutely after denervation.

Despite a neural differentiation protocol, embryonic stem cell derived motoneurons still carry a risk of tumorigenicity. Pre-treating with an anti-mitotic agent leads to survival and functional muscle reinnervation if performed within four weeks of denervation.

Introduction

Pathologies characterized by motoneuron (MN) death or axonal injury lead to muscle denervation and loss of motor function resulting in impairment in quality of life and longevity^{1,2}. Amyotrophic lateral sclerosis, spinal cord injuries, nerve root avulsion and plexus injuries result in motor dysfunction, either due to MN demise³ or irreversible denervation.

Restoring function to paralyzed muscles can be achieved with functional electrical stimulation provided the targeted muscle retains some innervation⁴. This strategy is far less effective if the targeted muscles lack innervation because large currents are required to directly activate denervated myofibres⁵. Consequently, we and others have explored the possibility of combining cell replacement therapy with electrical stimulation to restore function to permanently denervated muscles. Collectively, these studies have shown that MNs derived from embryonic ventral spinal cord cells⁶, embryonic stem (ES) cells⁷, or induced pluripotent stem (iPS) cells⁸, can functionally innervate denervated muscle fibres when transplanted into the peripheral nerve near the target muscle⁶⁻¹⁰. Although the innervating MNs do not fully restore pre-denervated contractile force, the level of force achieved when electrically stimulated is likely sufficient to provide meaningful function. For example, transplanted embryonic stem cell-derived motoneurons (ESCMNs) innervate denervated fibres and generate ~40% of the original contractile force when electrically stimulated, provided the cells were grafted immediately after muscle denervation⁷. These studies demonstrate proof of principle that cells transplanted into peripheral nerves can be stimulated to control muscle contraction.

In clinical practice, however, surgical interventions to improve denervation injuries are generally delayed to allow for spontaneous recovery¹¹. The same practice would apply if transplantation procedures were to be implemented. This delay could impact transplantation outcomes because

the local environment post denervation changes as time progresses¹². For translational purposes, it is therefore necessary to demonstrate that MNs can be transplanted after prolonged denervation and still provide functional innervation.

ES cells and iPS cells are alluring for cell replacement therapies: they can be expanded into large numbers and be directed to differentiate into specific neuronal types, including functional MNs^{13,14}. However, ES and iPS cells have been associated with the development of untoward outcomes following transplantation, such as the development of tumours¹⁵. Tumorigenesis likely results because the differentiation protocols lead only to enrichment of a specific cell type and do not completely eliminate pluripotent progenitor cells¹⁶. Consequently, strategies must be developed to eliminate the risk of tumorigenesis before these cell types can be used clinically.

Here we sought to establish a safe and effective means to transplant ESCMNs into peripheral nerves following prolonged denervation. We initially found that transplanting ESCMNs following a delay resulted in poor muscle reinnervation, and nearly half of the transplanted animals developed teratocarcinomas. We demonstrate that pre-treating the ESCMNs with the antimetabolic agent mitomycin C eliminated residual pluripotent cells following differentiation while sparing terminally differentiated MNs. Mitomycin C treatment prevented tumour formation, increased the success rate of functional engraftment, and led to functional reinnervation after prolonged injury similar to that seen following transplantation after acute denervation.

Materials And Methods

ESCMN Cell Preparation

HB9, a homeobox gene, is expressed in embryonic MNs early after differentiation from neuronal progenitors^{17,18}. The HBG3 transgenic mouse ES cell line linking the Hb9 promoter to an eGFP signal¹³ was generated on a mixed background⁷. An Hb9-eGFP ES cell line on a pure C57Bl6 background, denoted as HBGB6, was generously provided by Dr. Craig Cox (Jackson, ME, USA) and was also used as indicated. ESCMNs were generated by treating free-floating clusters of ES cells known as embryonic bodies (EB) with retinoic acid (1 μ M; Sigma) and smoothed agonist (500 nM; Enzo) as described previously^{7,13,14}. Motoneurons generated after five days of differentiation are still immature but fully differentiated and post-mitotic; electrophysiological characteristics will mature over four to seven days post-differentiation¹⁴. Differentiated EB were enzymatically dissociated in Tryple express (Gibco) with 0.01% (w/v) DNaseI (Sigma-Aldrich). The obtained single cell suspension was resuspended in DFK10 (as prepared in reference¹⁴) prior to use *in vivo* (see below) or *in vitro*. For *in vitro* experiments, 10⁵ dissociated cells were plated onto growth factor reduced matrigel (BD Biosciences)-coated glass coverslips (Fischer). *In vitro* cells were maintained in DFK10 supplemented with 10 ng/ml GDNF (Milipore) and 10 ng/ml CNTF (Chemicon). Media was changed every other day.

Surgery

All procedures were performed in accordance with protocols approved by the Dalhousie University Animal Care Committee, and conformed to the standards of the Canadian Council of Animal Care. Transplantation was performed as described previously⁷. In summary, under deep anaesthesia, the tibial nerve of 5-week old C57Bl6 mice (Charles River) was transected at mid-thigh level. Both nerve ends were ligated and the proximal end was buried into adjacent muscle

to prevent spontaneous reinnervation. ESCMN transplantation was done either immediately after transection or after a delay period of one, two, four, or eight weeks post transection. Ten thousand differentiated cells in 0.1 μ l containing 0.01% DNaseI, 20 μ g/ml CNTF and 10 μ g/ml GDNF were transplanted using a glass micro-pipette into the transected distal tibial nerve, which was subsequently proximally ligated to confine the transplanted cells inside the nerve. All distal branches except the medial gastrocnemius (MG) nerve branch were transected⁷.

Cross-anastomoses following acute or delayed (one week) denervation were performed as previously described¹⁹. Briefly, the mouse MG muscle was denervated by cutting the tibial nerve before a freshly cut common peroneal nerve was cross-sutured to its distal stump immediately or after a one week delay. This experimental paradigm allows study of the effects of prolonged muscle denervation independently from those of prolonged axotomy. Nerve ends were capped after transection for delayed reanastomosis with silicone tubes (Dow Corning). A 10-0 suture was used to perform an epineural apposition.

***In vitro* Electrophysiology**

The MG muscle together with the tibial nerve were harvested three months after transplantation as described previously⁷. Forces were measured with a force transducer (FT03, Grass Technologies) connected to an AC/DC strain gage amplifier (P122, Grass Technologies). EMG recordings were obtained with a polyethylene suction electrode (PE-190, Clay Adams) applied over the muscle mid-belly. EMG signals were amplified with a differential amplifier (EX4-400, Dagan) and bandpass filtered between 0.3 Hz to 3 kHz. A glass suction electrode was used to provide stimuli to the MG nerve via a square pulse stimulator (S88, Grass Technologies) isolated from ground by a constant current stimulus isolator (PSIU6, Grass Technologies). Signals were recorded via a Digidata 1320A, using Axoscope 9.2 software (Molecular Devices). Supra-maximal stimulation was performed at 1.5x the stimulus necessary to provide maximal

twitch force (usually 10V, 100 μ A, 0.2 ms). Tetanic stimulation was performed at 50 Hz sustained for 500 ms. Motor unit (MU) sizes were estimated by force increments^{7,20} obtained by stepwise increases of the stimulus. Motor unit number estimation was estimated by dividing the whole muscle twitch force by the averaged MU force obtained after seven force increments.

Tumour Tissue Harvesting And Culture

Upon palpable tumour formation from ESCMN transplantation, mice were anesthetized and the tumour was dissected under sterile conditions in terminal experiments. One half of the tumour was fixed and processed for paraffin embedding and thin slice hematoxylin and eosin histochemistry. The other half was kept for cell culture. Tissue was cut into minuscule fragments in a few drops of Ca²⁺/Mg²⁺ free HBSS. This slurry was transferred to a conical tube and washed multiple times before enzymatic digestion in trypsin-EDTA (1%-0.25%, Sigma-Aldrich) for 30 minutes at 37°C with constant agitation. The cell suspension was spun and washed with DMEM supplemented with 10% fetal calf serum (Gibco) followed by trituration, washing, resuspension and filtration over a 40 μ m cell filter (Millipore). In order to generate cell colonies, 10⁶ cells were plated onto a feeding layer of mitomycin C treated primary mouse embryonic fibroblasts (Stem Cell Technologies) in ES media as previously described^{13,14}, supplemented with 2x penicillin-streptomycin (Gibco) and 2.5 μ g/ml amphotericin B (Sigma-Aldrich). Media was changed daily for the first week because of abundant floating debris. Initial colonies were passaged on the fifth day in culture and every second day thereafter. Passaging of colonies was performed as described previously for mouse ES cultures.

Immunohistochemistry

Cells were fixed in 1% PFA in PBS for 20 minutes, followed by three washes in PBS, then perforated and blocked for 20 minutes in PBS-0.3% triton-X (v/v, Sigma-Aldrich) supplemented

with 10% donkey serum before incubating with primary antibodies. Cells were washed three times in PBS between each step. Nuclei were stained with Hoescht 33342 (Sigma-Aldrich) 0.1 $\mu\text{g/ml}$ for 20 minutes in PBS. Primary and secondary antibodies are described in Table 1.

Mitomycin C Treatment

To quantify the effect of mitomycin C on ESCMNs, differentiated EBs were incubated with increasing concentrations of mitomycin C (from 0.01 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$, Sigma-Aldrich) for 2 hours followed by three washes in HBSS, dissociation, and plating with neuroprogenitor cells (NPC) in an equal ratio on matrigel-coated coverslips as described elsewhere²¹. The presence of astrocytes from NPC was essential to maintain adhesion of ESCMNs to the coverslips for more than three days²². Coverslips were fixed after three, five or seven days *in vitro*. SSEA-1 immunofluorescence was used to identify undifferentiated cell colonies; ESCMN GFP signal was enhanced by immunofluorescence. ESCMNs and undifferentiated cell colonies were counted by selecting five random fields per coverslip with a 10x objective (area of 2.95 mm^2).

To quantify apoptotic cells by FACS after mitomycin C treatment, differentiated EBs were treated with 1 $\mu\text{g/ml}$ mitomycin C for two hours, washed, and incubated in suspension for 12 hours in DFK10 with 10 $\mu\text{g/ml}$ GDNF and CNTF. EBs were dissociated as per the ESCMN cell preparation protocol above. Annexin-V labelling (Life Technologies) was performed as per the manufacturer protocol with minor modifications. Dissociated cells were first immunolabelled with primary antibody against SSEA-1 (Table 1) for one hour at 4°C in cold annexin-binding buffer with constant agitation. After washing, annexin-V labelling was performed with secondary antibody added during the annexin-V conjugate incubation step. Cells were kept on ice until analyzed by FACS (BD FACS ArianIII) for the expression of eGFP, SSEA-1, and annexin-V.

Mitomycin C treatment for ESCMN transplantation was performed *in vitro* with 1 $\mu\text{g}/\text{ml}$ exposure for two hours prior to EB dissociation. EBs were washed three times with HBSS and dissociated as per the above protocols.

Imaging

Imaging was obtained with a Leica DMS microscope equipped with an Orca C4742-95 camera (Hamamatsu Photonics) and IP lab (version 4.01, Scanalytics Software). Colour images were obtained on an Axioplan II (Zeiss) microscope equipped with a colour Axiocam HRC camera (Zeiss). Confocal images were obtained on a Zeiss LSM710 confocal microscope running Zen software (Zeiss). Images were transferred to ImageJ (NIH) for analysis. Adobe Photoshop CS4 was used for contrast and brightness adjustment for publication purpose only.

Statistical Analysis

Results are presented as means \pm standard deviations. One-way ANOVA was used to compare electrophysiological data between experimental and control groups and between immediate and delayed experimental groups. Kurskal-Wallis tests and Dunn's multiple comparisons were applied to identify significant differences. Two-way ANOVA was used with mitomycin C experiments with Bonferroni multiple comparisons. Freeman-Halton extension of the Fisher's exact probability test was used for innervation success ratios. Statistics were calculated using Prism 5 (Graphpad).

Results

ESCMNs Transplanted After Prolonged Denervation Fail To Provide Innervation

Previous studies from our labs showed that, when transplanted into the distal stump of the cut tibial nerve immediately after transection, HBG3 ESCMNs innervate denervated MG muscles, and re-establish ~40% of their original contractile forces⁷. HBG3 ES cells were chosen for these studies because MNs derived from these stem cells express GFP upon neuronal differentiation allowing for easy morphological identification¹³. Here we sought to determine whether HBG3 ESCMNs successfully innervate muscle following delayed transplantation. In order to do so, we transplanted ESCMNs into transected tibial nerves of C57Bl6 mice one to eight weeks following nerve transection (10^5 cells/transplant). Three months after transplantation, the number of MG muscles innervated and whole muscle contractile forces were compared to those of animals transplanted at the time of nerve transection (denoted immediate in Figure 1). The proportion of animals with successful innervation decreased with increasing delays to transplantation (Table 2). In those muscles successfully innervated by cells transplanted at the time of nerve transection, the ESCMNs provided twitch (Figure 1A and B) and tetanic (Figure 1C and D) force recoveries of 63% and 55% respectively. Force recovery, however, was substantially reduced in the few instances when delayed transplantation led to muscle innervation, and resulted in 10% and 7% twitch and tetanic forces respectively after one week ($n = 2/6$ with reinnervation), 9% and 11% after two weeks ($n = 1/6$ with reinnervation), and 6% and 20%, respectively after four weeks ($n = 3/18$ with reinnervation; Figure 1A to D). No force production was seen if the ESCMNs were transplanted into the tibial nerve eight weeks after denervation ($n = 12$). By this time, the tibial nerve had degenerated to a clear structure only identifiable by the ligature placed during the original denervation surgery. Taken together, these results show that the success

rate of innervation is lower (Table 2), and the ability to re-establish contractile force is compromised when transplantation is delayed after denervation.

Given the reduction in innervation, we next asked whether the cells survive following delayed transplantation. There was no evidence of surviving cells when the transplantation was delayed by eight weeks. In contrast, a transplant nidus could be identified seven days after transplantation when the cells were grafted one to four weeks after nerve transection. In such cases, GFP⁺ neurites extended distally from the graft (Figure 1E). The lack of force recovery in the delayed transplants was therefore either due to the fact that the transplanted cells did not survive long enough to reinnervate muscle fibres or the fibres became refractory to innervation over time.

To address this point, we performed immediate and delayed MG muscle cross-anastomoses^{19,23} and recorded tetanic forces of the reinnervated muscles 3 months later. We found that the reinnervated MG muscles with the delayed anastomoses generated the same amount of twitch and tetanic forces as those muscles receiving an immediate cross-anastomosis (n = 5, p ≥ 0.25). This suggests that the early failure of reinnervation by ESCMNs was more likely related to some aspect of the transplanted cells rather than muscle-related factors.

Teratocarcinoma Formation Following Transplantation

We therefore turned to cellular factors that prevented successful reinnervation after delayed transplantation. Firstly, to determine if immunologic factors were limiting survival and innervation, we switched from the HBG3 ES cell line that was derived from a mixed 129Sv/C57Bl genetic background¹³ to a mouse stem cell line that was derived from a pure C57Bl6 background (denoted as HBGB6). Like MNs derived from the HBG3 cell line, ESCMNs derived from the HBGB6 stem cell line also express GFP. We found, however, that the use of

isogenic ESCMNs did not lead to improved regeneration. Instead, one week after immediate transplantation, GFP⁺ cells were seen in only two out of six mice. In a series of 17 transplants, only five (30%) were able to generate force at three months (3/10 immediate and 2/7 one week delayed transplants). Four of the remaining 12 transplanted animals had no sign of the graft, while eight generated tumours by one month (Figure 2A to C). The tumours were consistent with teratomas, with the presence of all three embryonic lineages on histology: ectoderm (neurons, Figure 2D), mesoderm (cartilage, Figure 2E) and endoderm (gastrointestinal mucosa, Figure 2F). Malignant histological features were also identified: mitotic figures (Figure 2G), nuclear atypia and hypercellularity (Figure 2H), and necrosis (Figure 2I), indicating the tumours were teratocarcinomas. The switch to a stem cell line that was isogenic to the recipient may have allowed tumours to expand because of an absence of immunological rejection as described by others¹⁶.

To demonstrate that pluripotent cells remained present within the tumours, we isolated and dissociated the tumours once they had become palpable in the mice (about one month) and cultured them on primary mouse embryonic fibroblasts in a manner similar to mouse ES cells. Tumour cells thus cultured formed colonies typical of stem cells and could be renewed for over one month when passaged every second day. These colonies were positive for the pluripotent markers SSEA-1, Oct-4A, and Sox2 (Figure 3A). Furthermore, these tumour-derived colonies could be differentiated into GFP⁺ MNs by treatment with retinoic acid and smoothed agonist as previously described for mouse ES cells^{13,14}. GFP expression was observed as early as two days *in vitro* (Figure 3B). This was seen as early as the third passage and the cells maintained these capacities even after 15 passages, representing one month in culture. In addition to GFP⁺ cells, β III-tubulin⁺/GFP⁻ cells were also present, demonstrating that these tumour-derived pluripotent cells, like ES cells^{13,14}, differentiated into more than one post-mitotic neuronal type

(Figure 3C). Taken together, these results indicate that the observed tumours contained pluripotent cells that could be differentiated into a number of neuron types, including MNs. Furthermore, this shows that the development of malignant teratocarcinomas is a substantial risk in mice when isogenic cells are used for transplantation.

Pluripotent Cells Remain Even After Differentiation Of Stem Cells

We next examined the extent to which pluripotent cells remained when HBGB6 ES cells were differentiated into MNs using retinoic acid and smoothed agonist. Figure 4A shows that following differentiation, ESCMN cultures contained cells expressing the pluripotent markers SSEA-1, Oct4A, and Sox2. As expected, none of the GFP⁺ MNs expressed these markers. Using FACS analysis we found that $6 \pm 2\%$ ($n = 3$) of the cells expressed SSEA-1 after differentiation protocol. Once plated, all wells ($n > 12$) with differentiated ESCMNs formed SSEA-1 expressing cell colonies as early as three days *in vitro*. These data indicate that residual pluripotent cells persisted within differentiated ESCMN cultures, and suggest that these pluripotent cells likely caused the malignancies.

Treatment With Mitomycin C Eliminates Pluripotent Cells

Our next strategy was based on the reasoning that since neurons are mitotically inactive, pluripotent cells could be eliminated while neurons preserved by using the alkylating agent mitomycin C. We therefore treated differentiated EBs with $1 \mu\text{g/ml}$ mitomycin C for two hours and immunolabelled the cells with SSEA-1 and the apoptotic marker annexin-V. SSEA-1⁺ cells were isolated using FACS and then further analyzed for their expressing of annexin-V and GFP. This showed that the proportion of SSEA-1⁺ cells expressing annexin-V was twice as high in the mitomycin C treated group (Figure 4B, mitomycin C lower right quadrant) compared to control (Figure 4B, Control lower right quadrant). To test whether this increase in annexin-V was

associated with a reduction in the number of pluripotent cells, we treated the differentiated EBs with mitomycin C (1 $\mu\text{g/ml}$, 2 hours), and then cultured them for one week. This eliminated pluripotent cells, as demonstrated by the absence of SSEA-1⁺ cells in the mitomycin C-treated cultures (Figure 4C). Concentrations of at least 1 $\mu\text{g/ml}$ of mitomycin C were required to effectively eliminate SSEA-1⁺ cells from the cultures (Figure 4D). Thus, mitomycin C was found to be effective at eliminating undifferentiated pluripotent cells from EBs.

We next asked whether mitomycin C was toxic to MNs. Three days after differentiation and dissociation, there were fewer ESCMNs treated with mitomycin C compared to untreated controls, but by five days, there was no difference in ESCMN numbers (Figure 4E). At all time points, however, survival of MNs was significantly decreased when EBs were treated with ≥ 5 $\mu\text{g/ml}$ mitomycin C. Thus, mitomycin C treatment of 1-2 $\mu\text{g/ml}$ was effective at eliminating pluripotent cells while preserving differentiated MNs.

Mitomycin C Prevents The Formation Of Teratocarcinoma After Transplantation And Improves Reinnervation

Given that incubation with mitomycin C led to elimination of pluripotent cells, we next tested whether mitomycin C-treated HBGB6 ESCMNs formed tumours after transplantation into isogenic mice. ESCMNs treated with mitomycin C did not engraft after immediate transplantation (n = 6). However, when mitomycin C-treated ESCMNs were transplanted one to four weeks after tibial nerve transection, none (0/20) of the animals developed tumours, indicating that mitomycin C was effective at preventing the formation of teratocarcinomas.

We next determined whether the treated ESCMNs survived and innervated muscle when transplanted into the peripheral nerve of mice. Treating the cells prior to transplantation with mitomycin C resulted in a greater number of animals with successful innervation after delayed

transplantation (six out of eight animals following one week delay; three out of six animals after two weeks delay; and two out of six animals after four weeks delay; Table 3). Furthermore, twitch and tetanic forces after three months were similar to those found after immediate transplantation of non-mitomycin treated HBGB6 ESCMNs (Figure 5A to D). The average MU force was increased compared to normal MU, but was not statistically different between transplant groups (Figure 5E). The motor unit number estimation was ~13 (Figure 5F) similar to the number we reported previously for immediate transplantation⁷. Together, these results demonstrate that incubating ESCMNs with the antimetabolic agent mitomycin C not only prevented tumour formation, but also led to improved engraftment and innervation following delays between denervation and transplantation.

Discussion

In developing a clinically relevant model of cell transplantation for denervation injury, we found that muscle fibre innervation decreased dramatically with increasing delays between nerve transection and ESCMN transplantation. Concurrently with this, we found that about 50% of animals developed teratocarcinomas, which arose from residual pluripotent cells within the graft. We addressed tumorigenesis by pre-treating the cultures with the antimetabolic agent mitomycin C, and found that this prevented cancer formation and led to significantly improved muscle innervation from transplanted ESCMNs.

Motor Force Restoration

Following denervation, return of innervation can occur within a finite time window (five weeks in mice²⁴, 12 weeks in rats¹⁹, 12 to 18 months in humans²⁵), outside of which functional recovery is poor^{19,23,26}. In the absence of innervation, muscle contraction cannot be efficiently restored⁵.

To this end, transplantation of MNs into either the spinal cord grey matter or the peripheral nervous system has been investigated and has demonstrated that transplanted MNs are able to reinnervate muscle fibres^{27,28}. Force generation in our transplants performed either immediately⁷ or after prolonged denervation (with mitomycin C pre-treatment) recovered to about half of control forces, a finding that is consistently demonstrated after immediate transplantation by other groups as well^{7-10,29-31}. We estimated that after transplantation, ~15 MUs innervated the MG, whereas the mouse MG normally contains ~50 MUs^{7,32}. Given the sprouting capacity of MNs (up to five times their native innervation ratio³³), it would be expected that all muscle fibres would be reinnervated, and that close to normal force should be restored. There are two possible explanations as to why this was not the case: (1) muscle fibre types changed and/or (2) ESCMNs have a more limited capacity to form enlarged MUs compared to endogenous MNs.

Support for the former possibility comes from our previous study⁷, which showed a dramatic increase in slow muscle fibres in the mouse MG following ESCMN innervation. Because slow fibres are smaller and less powerful than fast fibres, this conversion would result in smaller whole muscle force³⁴. While we have no evidence that there is a cell-autonomous reason for ESCMNs to have impaired capacity to form enlarged MUs, the lack of activity resulting from their reduced microcircuit environment may limit their ability to form the number of axonal branches required to expand their innervation ratio³⁵. This inability of ESCMNs to form enlarged MUs would result in a large number of muscle fibres remaining denervated, which in turn, would lead to a loss in contractile forces. Thus, the smaller forces generated by transplants likely resulted from fewer MNs combined with both a conversion in muscle fibre type and smaller MUs.

Timing Of Transplantation

We found that a delay of transplantation beyond one week resulted in worse outcomes^{30,36}. At one week, the inflammatory environment resulting from the transection has likely transformed to a restorative milieu rich in neurotrophic factors, axonal growth promoting substrates and adhesion molecules produced by activated Schwann cells^{30,37}. Given that the rate of cellular death in the grafts is highest at the time of transplantation³⁸, these survival signals may be essential to sustain initial transplant survival. In fact, during development, Schwann cells provide adhesion molecules and trophic support for embryonic MN survival during the critical period of programmed cell death³⁹. For these reasons, mitomycin C-treated ESCMNs transplanted immediately likely failed to engraft; Schwann cells had not yet proliferated to provide adhesive and trophic support and glial cells normally present in EBs would have been ablated by the mitomycin C treatment. In fact, ESCMNs treated with mitomycin C always died by the fifth day *in*

vitro if not co-cultured with glial cells, a finding common to embryonic neurons²² even in the absence of mitomycin C treatment.

Tumour Prevention

When considering translation of stem cell therapies, it is crucial to be able to prevent cancer formation⁴⁰. The formation of teratocarcinomas from embryonic tissues is mostly dependent on the transplanted environment and the host immune system⁴³. Our initial use of an allogeneic stem cell line{Yohn:2008hx} may have triggered the host immune system to reject residual pluripotent cells. Our introduction of an isogenic HBGB6 cell line could thus have contributed to tumorigenesis. As few as two undifferentiated stem cells in two million non-neoplastic cells can form tumours in 60% of transplants; this rate reached 100% of transplants when 20 undifferentiated stem cells were transplanted⁴¹. Given that current sorting techniques are limited to the detection of 1 in 10 000⁴², pre-sorting of cells is currently inadequate, although future sorting techniques may eventually be able to eliminate all pluripotent cells. The use of mitomycin C – via a short two-hour exposure time prior to transplantation – prevented early tumour formation by eliminating tumorigenic cells.

Translational Considerations

When considering cell-based therapies for MN loss, it is pragmatic to first consider proximal peripheral nerve and plexus injuries: these lead to significant deficits and are associated with limited functional recovery⁴⁴. Commonly, the approach to nerve injury is to delay invasive interventions in order to identify spontaneous recovery¹¹. Consequently, any cell-based therapy would be delayed. We demonstrate that delayed transplantation of ESCMNs is possible and effective if tumour formation is prevented.

Because the neurons in this model are transplanted into the periphery, there is no connection with the central nervous system, and thus no voluntary control. To obtain control, this technique could be combined with functional electrical stimulators to activate the transplanted neurons. Alternatively, ESCMNs could perhaps be considered as “placeholders”, preserving muscle fibre innervation until endogenous axons return.

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Ethics

The Dalhousie Animal Use Committee, in accordance with the standards of the Canadian Council of Animal Care, approved all procedures.

Author Contributions

PM, VRF, and RMB contributed to the conception and design of the study, PM acquired and analyzed the data, and PM, VRF, and RMB wrote the manuscript.

Conflicts Of Interest

Authors have declared no conflict of interest.

References

1. Bromberg MB. Quality of life in amyotrophic lateral sclerosis. *Phys Med Rehabil Clin N Am* 2008;19(3):591–605.
2. Boakye M, Leigh BC, Skelly AC. Quality of life in persons with spinal cord injury: comparisons with other populations. *J Neurosurg Spine* 2012;17(1 Suppl):29–37.
3. Carlstedt T, Cullheim S. Spinal cord motoneuron maintenance, injury and repair. *Prog Brain Res* 2000;127:501–514.
4. Prochazka A, Mushahwar VK, McCreery DB. Neural prostheses. *J Physiol (Lond)* 2001;533(Pt 1):99–109.
5. Popovic D, Gordon T, Rafuse VF, Prochazka A. Properties of implanted electrodes for functional electrical stimulation. *Ann Biomed Eng* 1991;19(3):303–316.
6. Thomas CK, Erb DE, Grumbles RM, Bunge RP. Embryonic cord transplants in peripheral nerve restore skeletal muscle function. *J Neurophysiol* 2000;84(1):591–595.
7. Yohn DC, Miles GB, Rafuse VF, Brownstone RM. Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy. *Journal of Neuroscience* 2008;28(47):12409–12418.
8. Toma JS, Shettar BC, Chipman PH, et al. Motoneurons derived from induced pluripotent stem cells develop mature phenotypes typical of endogenous spinal motoneurons. *Journal of Neuroscience* 2015;35(3):1291–1306.
9. Erb DE, Mora RJ, Bunge RP. Reinnervation of adult rat gastrocnemius muscle by embryonic motoneurons transplanted into the axotomized tibial nerve. *Exp Neurol* 1993;124(2):372–376.
10. Bryson JB, Machado CB, Crossley M, et al. Optical Control of Muscle Function by Transplantation of Stem Cell-Derived Motor Neurons in Mice. *Science* 2014;344(6179):94–97.
11. Kline DG. Physiological and clinical factors contributing to the timing of nerve repair. *Clin Neurosurg* 1977;24:425–455.
12. Scheib J, Hoke A. Advances in peripheral nerve regeneration. *Nat Rev Neurol* 2013;9(12):668–676.
13. Wichterle H, Lieberam I, Porter JA, Jessell TM. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 2002;110(3):385–397.
14. Miles GB, Yohn DC, Wichterle H, et al. Functional properties of motoneurons derived from mouse embryonic stem cells. *Journal of Neuroscience* 2004;24(36):7848–7858.

15. Kiuru M, Boyer JL, O'Connor TP, Crystal RG. Genetic Control of Wayward Pluripotent Stem Cells and Their Progeny after Transplantation. *Stem Cell* 2009;4(4):289–300.
16. Hentze H, Graichen R, Colman A. Cell therapy and the safety of embryonic stem cell-derived grafts. *Trends in Biotechnology* 2007;25(1):24–32.
17. Arber S, Han B, Mendelsohn M, et al. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. *Neuron* 1999;23(4):659–674.
18. Thaler J, Harrison K, Sharma K, et al. Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. *Neuron* 1999;23(4):675–687.
19. Fu SY, Gordon T. Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. *J Neurosci* 1995;15(5 Pt 2):3886–3895.
20. Mccomas AJ, Fawcett PRW, Campbell MJ, Sica REP. Electrophysiological estimation of the number of motor units within a human muscle. *J Neurol Neurosurg Psychiatr* 1971;34(2):121–131.
21. Rafuse VF, Soundararajan P, Leopold C, Robertson HA. Neuroprotective properties of cultured neural progenitor cells are associated with the production of sonic hedgehog. *NSC* 2005;131(4):899–916.
22. Wang XF, Cynader MS. Effects of astrocytes on neuronal attachment and survival shown in a serum-free co-culture system. *Brain Res. Brain Res. Protoc.* 1999;4(2):209–216.
23. Fu SY, Gordon T. Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy. *J Neurosci* 1995;15(5 Pt 2):3876–3885.
24. Sakuma M, Gorski G, Sheu S-H, et al. Lack of motor recovery after prolonged denervation of the neuromuscular junction is not due to regenerative failure. *Eur J Neurosci* 2015;;n/a–n/a.
25. Robinson LR. Traumatic injury to peripheral nerves. *Muscle Nerve* 2000;23(6):863–873.
26. Irintchev A, Draguhn A, Wernig A. Reinnervation and recovery of mouse soleus muscle after long-term denervation. *Neuroscience* 1990;39(1):231–243.
27. Nógrádi A, Szabó A. Transplantation of embryonic neurones to replace missing spinal motoneurones. *Restor Neurol Neurosci* 2008;26(2-3):215–223.
28. Deshpande DM, Kim Y-S, Martinez T, et al. Recovery from paralysis in adult rats using embryonic stem cells. *Ann Neurol* 2006;60(1):32–44.
29. Cui L, Jiang J, Wei L, et al. Transplantation of Embryonic Stem Cells Improves Nerve Repair and Functional Recovery after Severe Sciatic Nerve Axotomy in Rats. *Stem Cells* 2008;

30. Grumbles RM, Wood P, Rudinsky M, et al. Muscle reinnervation with delayed or immediate transplant of embryonic ventral spinal cord cells into adult rat peripheral nerve. *Cell transplantation* 2002;11(3):241–250.
31. Grumbles RM, Sesodia S, Wood PM, Thomas CK. Neurotrophic factors improve motoneuron survival and function of muscle reinnervated by embryonic neurons. *J Neuropathol Exp Neurol* 2009;68(7):736–746.
32. Mohajeri MH, Figlewicz DA, Bohn MC. Selective loss of alpha motoneurons innervating the medial gastrocnemius muscle in a mouse model of amyotrophic lateral sclerosis. *Exp Neurol* 1998;150(2):329–336.
33. Rafuse VF, Gordon T, Orozco R. Proportional enlargement of motor units after partial denervation of cat triceps surae muscles. *J Neurophysiol* 1992;68(4):1261–1276.
34. Tötösy de Zepetnek JE, Zung HV, Erdebil S, Gordon T. Innervation ratio is an important determinant of force in normal and reinnervated rat tibialis anterior muscles. *J Neurophysiol* 1992;67(5):1385–1403.
35. Rutishauser U, Landmesser L. Polysialic acid on the surface of axons regulates patterns of normal and activity-dependent innervation. *Trends Neurosci* 1991;14(12):528–532.
36. Lin S, Xu L, Hu S, et al. Optimal time-point for neural stem cell transplantation to delay denervated skeletal muscle atrophy. *Muscle Nerve* 2013;47(2):194–201.
37. deLapeyrière O, Henderson CE. Motoneuron differentiation, survival and synaptogenesis. *Curr Opin Genet Dev* 1997;7(5):642–650.
38. Sortwell CE, Pitzer MR, Collier TJ. Time Course of Apoptotic Cell Death within Mesencephalic Cell Suspension Grafts: Implications for Improving Grafted Dopamine Neuron Survival. *Exp Neurol* 2000;165(2):268–277.
39. Banks GB, Noakes PG. Elucidating the molecular mechanisms that underlie the target control of motoneuron death. *Int J Dev Biol* 2002;46(4):551–558.
40. Bretzner F, Gilbert F, Baylis F, Brownstone RM. Target populations for first-in-human embryonic stem cell research in spinal cord injury. *Cell Stem Cell* 2011;8(5):468–475.
41. Lawrenz B, Schiller H, Willbold E, et al. Highly sensitive biosafety model for stem-cell-derived grafts. *Cytotherapy* 2004;6(3):212–222.
42. Geens M, Van de Velde H, De Block G, et al. The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. *Hum. Reprod.* 2006;22(3):733–742.
43. Damjanov I. Teratocarcinoma: neoplastic lessons about normal embryogenesis. *Int J Dev Biol* 1993;37(1):39–46.
44. Giuffre JL, Kakar S, Bishop AT, et al. Current Concepts of the Treatment of Adult Brachial Plexus Injuries. *J Hand Surg Am* 2010;35(4):678–688.

Figure Legends

Figure 1 – Delayed Transplantation Of ESCMNs Leads To Poor Force Recovery Three Months Post-Transplantation

(A) Example of twitch forces of the MG evoked by stimulation of the tibial nerve *ex vivo* in surgically naive mice (no surgery), immediately transplanted mice (immediate), and delayed transplanted mice (1 and 8 weeks). (B) Quantification of twitch forces. (C) Example of 50 Hz tetanic forces evoked in the same groups as (A). (D) Quantification of 50 Hz tetanic forces. (E) Growth of neurites after delayed (1 and 4 weeks) transplantation was visible along the nerves at 7 days after transplantation. Transplant site delineated by the dotted line. ns: non-significant, ** $p = 0.006$, *** $p = 0.0006$, **** $p < 0.0001$, multiple comparisons between immediate and delayed transplants are non-significant for both twitch and tetanic forces.

Figure 2 – Isogenic Transplants Lead To Formation Of Teratocarcinomas

Macroscopic (A-C) appearance of tumour. This first tumour appeared rapidly and unexpectedly. Once aware of this issue, we ensured that no further tumours grew larger than a palpable size of 1 cm. Microscopic (D-I) images of tumours originating from transplanted ESCMNs. All three germ lineages were present in tumours consistent with the formation of a teratomatous tumours: epidermal lineage (neuron: D), mesodermal lineage (cartilage: E) and endodermal lineage (ciliated glandular epithelium with goblet cells: F). Characteristics of a malignant teratoma (teratocarcinoma): high mitotic rate (G), hypercellularity with nuclear atypia (H), and intra-tumoral necrosis (I).

Figure 3 – Pluripotent Cells Isolated From Teratocarcinomas Generated From Transplanted ESCMNs Can Be Differentiated Into MNs *In vitro*

(A) Pluripotent cells isolated from dissociated teratocarcinoma tissue formed colonies expressing the stem cell markers SSEA-1, Sox2, and Oct4A. Hoescht staining was used to visualize individual nuclei. (B) Following treatment with retinoic acid and a smoothen agonist, EBs generated from teratocarcinoma-derived cells contain GFP⁺ MNs after 2 and 7 days *in vitro*. (C) Dissociated and plated EBs that were treated for 5 days *in vitro* with retinoic acid and a smoothened agonist, contained GFP⁺ MNs and β III-tubulin⁺ cells that were GFP⁻ after 2 days *in vitro*.

Figure 4 – Residual Pluripotent Cells Post ESCMN Differentiation Are Sensitive To Mitomycin C

(A) Dissociated differentiated EBs grown for five days *in vitro* on matrigel demonstrate the formation of colonies expressing pluripotent markers (SSEA-1, Oct4A, Sox2) in the absence of LIF and PMEF. Scale bar 100 μ m. (B) FACS sorting of SSEA-1⁺ cells from dissociated and annexin-V immunolabelled EBs (previously treated with retinoic acid and a smoothened agonist) without and with pre-treatment with mitomycin C (1 μ g/ml for 2 hours) 12 hours prior to sorting. The shift to the right indicates that the majority of SSEA-1⁺ cells expressed annexin-V (but not GFP) after mitomycin C exposure. (C) Seven days following treatment with 1 μ g/ml mitomycin C, dissociated EBs did not contain any colonies of SSEA-1⁺ cells (right), but these pluripotent cells were present in untreated EBs (left). Scale bar 100 μ m. (D) Dose response of SSEA-1⁺ colonies seven days *in vitro* after mitomycin C treatment. No colonies were found when EBs were treated with mitomycin C concentrations of 1 μ g/ml or above. * p < 0.05 compared to controls. (E) Toxicity of mitomycin C on ESCMNs showing statistically significant effects with concentrations of 5 μ g/ml or greater at all times points compared to controls. ** p < 0.01 compared with controls of the same time point, by two-way ANOVA and Bonferonni multiple comparisons.

Figure 5 – Improved Reinnervation After Delayed Transplantation Of ESCMNs Treated With Mitomycin C

(A-B) Twitch force and (C-D) 50 Hz tetanic force of the MG in delayed transplantation (1, 2, and 4 weeks delay). No group is statistically significant from Immediate transplant by Kruskal-Wallis test. (E) Average MU force from transplanted mice obtained by force gradation with increasing stimulus. (F) Motor unit number estimation: there was no statistical difference between groups.

*Forces from immediate transplants are those of HBGB6 ESCMNs not treated with mitomycin C and are being shown for reference only; mitomycin C-treated ESCMNs transplanted immediately after nerve transection did not innervate (n = 6).

Table 1 – Immunohistochemistry antibodies

Primary Antibodies						
Antiserum	Host	Subtypes	Dilutions	Incubation	Sources	Cat. Number
GFP	Rabbit	IgG	1:1000	1 hr	Millipore	AB3080
GFP	Sheep	IgG	1:1000	O/N	Novus Biologicals	NB100-62622
TUJ1	Mouse	IgG2a	1:1000	1 hr	Covance	MMS-435P
SSEA-1*	Mouse	IgM	1:1000**	1 or 72 hrs	Cell Signaling	4744
Sox2	Mouse	IgG1	1:100	72 hrs	Cell Signaling	4900
Oct4A	Rabbit	IgG	1:100	72 hrs	Cell Signaling	2840
Secondary Antibodies						
Antiserum	Host	Conjugates	Dilutions	Incubation	Sources	Cat. Number
Anti-Rabbit IgG	Donkey	AF488	1:500	1 hr	Life Technologies	A-21206
Anti-Sheep IgG	Donkey	AF488	1:500	O/N	Life Technologies	A-11015
Anti-Mouse IgG	Donkey	AF546	1:500	1 hr or O/N	Life Technologies	A10036
Anti-Mouse IgM	Goat	AF546	1:500*	1 hr or O/N	Life Technologies	A-21045
Anti-Rabbit IgG	Donkey	AF647	1:500	O/N	Life Technologies	A-31573

*Best staining results obtained without cell perforation at 1:1000 for 1 hour; 72 hours incubation at 4°C was only performed when combined with Sox2 and Oct4A staining.

**Used at 1:100 for 20 minutes for FACS experiments.

Table 2 – Innervation of MG by transplanted ESCMNs after prolonged denervation

Transplantation delay	Animals with MG contraction (%)	Transplanted animals
Immediate	3 (50%)	6
1 week delay	2 (33%)	6
2 weeks delay	1 (17%)	6
4 weeks delay	3 (17%)	18
8 weeks delay	0 (0%)	12

Successful innervation by transplanted HBG3 ESCMNs was defined by the presence of MG contraction. Fischer exact t-test $p = 0.04$

Table 3 – Innervation of MG by transplanted mitomycin-treated ESCMNs after prolonged denervation

Transplantation delay	Animals with MG contraction (%)	Transplanted animals
Immediate	0 (0%)	6
Immediate*	3 (50%)	6
1 week delay	6 (75%)	8
2 weeks delay	2 (33%)	6
4 weeks delay	2 (25%)	10
8 weeks delay	ND	ND

*Immediate group representing HBGB6 ESCMNs transplanted without mitomycin treatment. Mitomycin-treated ESCMNs transplanted immediately after denervation (n = 6) did not generate contraction. Fischer exact t-test p = 0.12

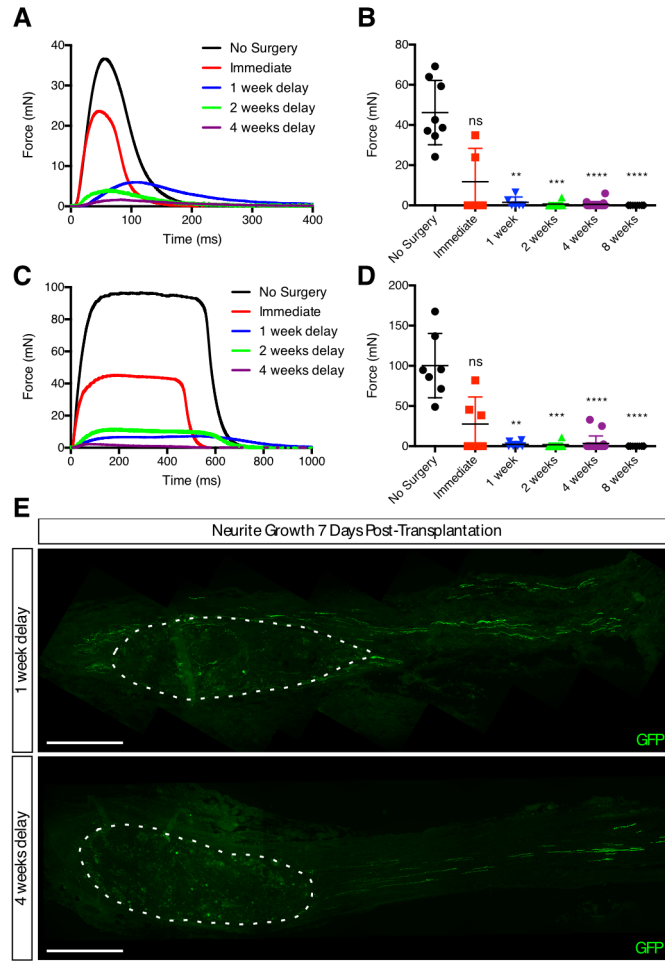


Figure 1 – Delayed transplantation of ESCMNs leads to poor force recovery 3 months post-transplantation

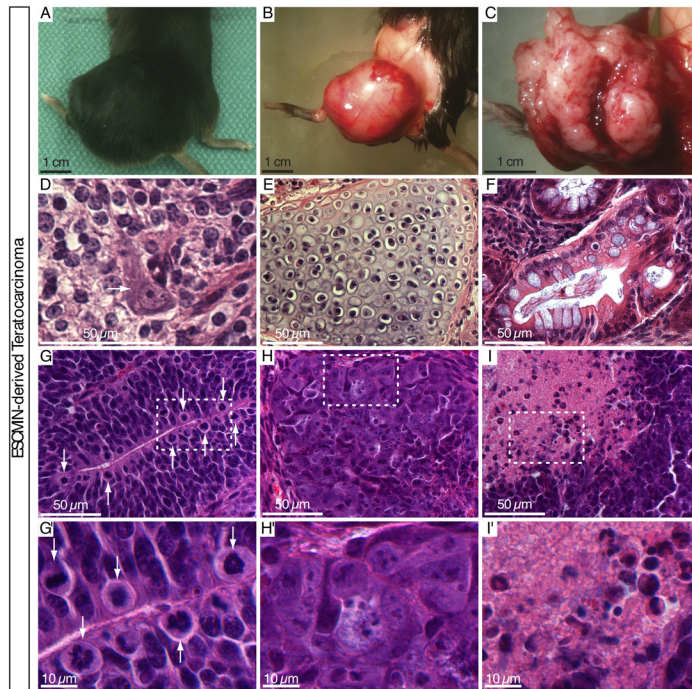


Figure 2 – Isogenic transplants lead to formation of teratocarcinomas

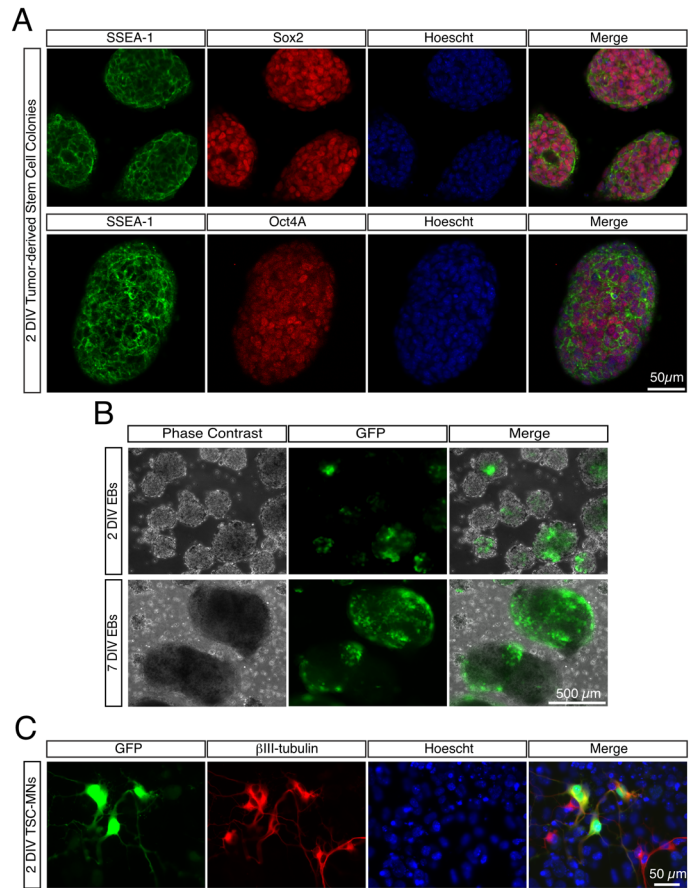


Figure 3 – Pluripotent cells isolated from teratocarcinomas generated from transplanted ESCMNs can be differentiated into MNs *in vitro*

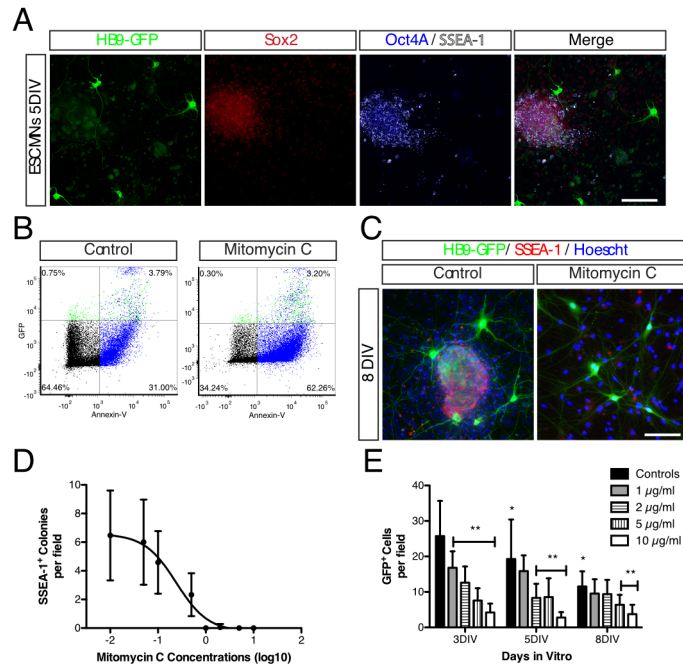


Figure 4 – Residual pluripotent cells post ESCMN differentiation are sensitive to mitomycin C

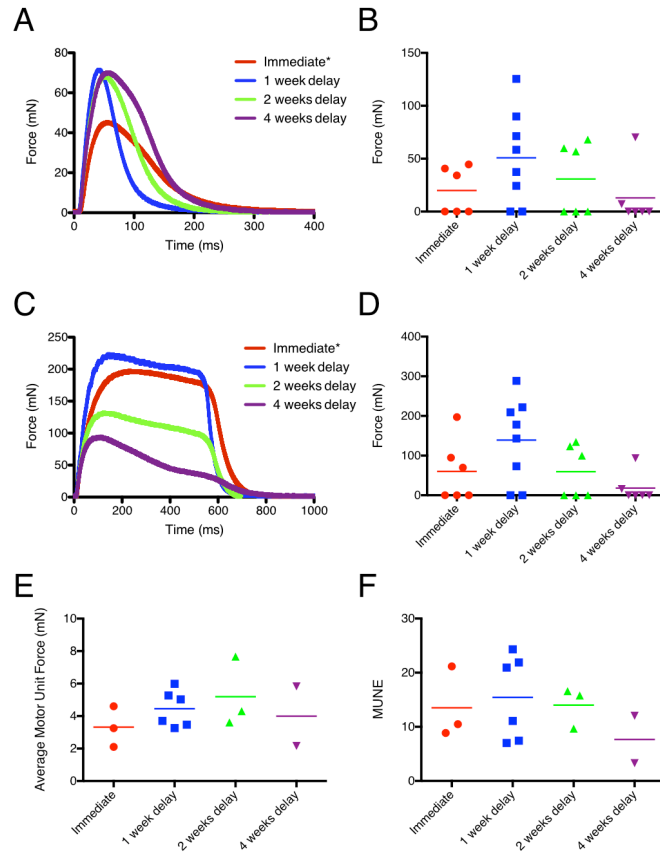


Figure 5 – Improved reinnervation after delayed transplantation of ESCMNs treated with mitomycin C

Chapter Three: Direct Optical Activation Of Skeletal Muscle Fibres Efficiently Controls Muscle Contraction And Attenuates Denervation Atrophy

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Abstract

Neural prostheses can restore meaningful function to paralyzed muscles by electrically stimulating innervating motor axons but fail when muscles are completely denervated, as seen after ALS or peripheral nerve and spinal cord injuries. Here we show that channelrhodopsin-2 can be expressed within the sarcolemma and T-tubules of skeletal muscle fibres in transgenic mice. This expression pattern allows for optical control of muscle contraction with comparable forces to nerve stimulation. Force can be controlled by varying light pulse intensity, pulse duration, or pulse frequency. Light-stimulated muscle fibres depolarize proportionally to light intensity and duration. Denervated triceps surae muscles transcutaneously stimulated optically on a daily basis for 10 days show a significant attenuation in atrophy resulting in significantly greater contractile forces compared to chronically denervated muscles. Together, this study shows that channelrhodopsin-2/H134R can be used to restore function to permanently denervated muscles and reduce pathophysiological changes associated with denervation pathologies.

Introduction

Spinal cord injuries (SCIs) and peripheral nerve injuries, such as a brachial plexus avulsion, cause severe motor deficits that ultimately impact the physical, psychological, and social well-being of those affected. Restoring meaningful function to specific muscle groups, such as those controlling hand grip, can increase independence and improve overall quality of life¹.

Restoration of function to paralyzed hand muscles has been achieved by combining functional electrical stimulation (FES) with neural prostheses by electrically stimulating the innervating motor nerve^{2,3}. Neural prostheses do not function if the targeted muscles are completely denervated. Unfortunately, SCIs and peripheral nerve injuries often result in complete and permanent muscle denervation because their associated motor neurons either died from the injury⁴ or fail to restore innervation in time. The only means to restore function to permanently denervated muscles is through cell replacement therapy (i.e. motor neuron transplantations) or by a different form of exogenous activation.

Motor neurons derived from embryonic ventral cord cells⁵, embryonic stem (ES) cells⁶, or induced pluripotent stem (iPS) cells⁷ have been transplanted into the peripheral nerve environment near the motor nerve entry point of completely denervated muscles. In all cases, transplanted motor neurons restored some motor functions by reinnervating muscle fibres. Furthermore, when electrically stimulated, the transplanted neurons evoked appreciable force contractions (up to 50% of control values). Recently, Bryson and colleagues⁸ extended on these studies by generating genetically modified ES cell-derived motor neurons expressing the light-sensitive ion channel, channelrhodopsin-2 (ChR2)⁹. Optical stimulation of the transplanted ChR2 motor neurons generated contractile forces equal to 12% of control values⁸.

Studies such as these support the development of strategies to restore function to denervated muscles by combining FES technology with the transplantation of motor neurons derived from pluripotent cells. However, two portentous issues preclude introducing this technology clinically. First, several studies reviewed by Knoepfler have shown that teratomas can form from residual pluripotent cells in the transplanted population even when directed to differentiate prior to transplantation¹⁰. Second, it is well established that transected nerves and denervated muscles become refractory to growth and reinnervation over time¹¹⁻¹³. Thus, unless motor neurons are grafted shortly after an irreversible denervation injury, functional motor recovery will likely remain poor.

To evade these issues we tested whether direct expression of ChR2 in skeletal muscle enables efficient optical control of muscle force and function and whether denervated muscles can be chronically activated after complete nerve transection. Optical control of muscle contraction could be achieved with an off-the-shelf blue LED by varying light pulse intensity, pulse duration and pulse frequency and generated comparable forces to neural-evoked contractions. Intracellular potentials were investigated to determine the properties of ChR2-induced depolarizations with and without action potential propagation along the sarcolemma. Muscle fibre depolarization by ChR2 activation is function of light intensity and duration. In the presence of myosin inhibitors, a single action potential was generated upon ChR2 activation followed by a rebound and plateau. Finally, after daily optical stimulation of denervated muscle, muscle fibre atrophy was attenuated and contractile force partially spared. Our proof of principle study demonstrates the capacity for fine optical control of functional muscle contraction and sparing of muscle atrophy after denervation.

Materials And Methods

Mouse Strain

Sim1^{cre/+} mice, described elsewhere⁴⁵, were crossed with Ai32 mice obtained from Jackson Laboratory (strain B6; 129S-*Gt(ROSA)26Sor^{tm32(CAG-COP4*H134R/EYFP)Hze/J}*, stock number 012569) to generate Sim1-Ai32 mice. These mice express a modified ChR2/EYFP fusion protein where the opsin protein harbors a gain-of-function H134R substitution permitting to generate larger photocurrents¹⁶ under blue light (450-490 nm) stimulation. *Sim1^{cre/+}* animals were genotyped with primers targeting the Cre-recombinase sequence⁴⁶. ChR2 animals were genotyped with the following primers (5' to 3'): forward ACA TGG TCC TGC TGG AGT TC, reverse GGC ATT AAA GCA GCG TAT CC.

In-Vitro Soleus Muscle Isometric Tension

Adult female Sim1-Ai32 mice were used throughout this study. All procedures were approved by the ethics committee at Dalhousie University and followed the Canadian Tri-Council guidelines for laboratory animals. Under isoflurane anesthesia, animals were euthanized by cervical dislocation and the left soleus muscle (n = 5) was quickly dissected out in ice-cold oxygenated (5% CO₂/95% O₂) Tyrode's solution (125 mM NaCl, 24 mM NaHCO₃, 5.37 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂ and 5% dextrose) before transferring to a Sylgard-coated (Dow Corning) recording chamber perfused with oxygenated Tyrode's solution at room temperature. The distal tendon was attached to a force transducer (FT03, Grass Technologies) with a silk tie to measure isometric contraction. A glass suction electrode (BF120-90-10, WPI) was used to deliver electrical stimulation to the nerve via an S88 stimulator (Grass Technologies). The stimulus was isolated from ground by a stimulus isolation unit (PSU6, Grass Technologies) delivering a monophasic supramaximal stimulus, defined as 1.5 times the stimulus for maximal

twitch force ($\sim 100\mu\text{A}$), with a pulse duration of 0.2 ms. EMG was recorded with a polyethylene suction electrode (PE-190, Clay Adams) positioned on the soleus muscle belly. EMGs were amplified with a 10x pre-amplifier (4001 differential headstage, Dagan Corporation) and further 10x amplified and bandwidth filtered between of 3Hz and 3kHz (EX4-400, Dagan Corporation). EMG amplitude was measured as the maximum excursion between the positive and negative phases of the waveform (as defined by isoelectric crossings) while EMG duration was quantified as the time between the onset and offset of the first and second phase of the EMG potential. Force and EMG responses were acquired at 10kHz using a Digidata 1322A A/D board (Axon Instruments) and Axoscope 9.2 software (Axon Instruments). Optical stimulation was performed with a blue (470 nm) Rebel Star O Drop-in LED from Luxeon Star (Quadica Development Inc.) positioned 1 cm above the muscle. The LED current was controlled with an AC/DC converter connected to a solid-state relay triggered with a S88 stimulator (Grass Technologies). The LED irradiance was measure with a photodetector amplifier (PDA1, World Precision Instrument) connected to a silicone planar photodiode (VISD, Word Precision Instruments) positioned at 1cm distance from the light source.

Drug Treatments

Muscle contractions were determined in the presence of the following drugs: $10\mu\text{M}$ d-tubocurarine (Sigma), 500 nM tetrodotoxin (Abcam), $1\mu\text{M}$ μ -conotoxin (Alomone Labs), $50\mu\text{M}$ *N*-benzyl-*p*-toluene sulphonamide (BTS; Tocris Bioscience) and $10\mu\text{M}$ blebbistatin (Sigma).

Intracellular Myofibre Electrophysiology

Intracellular muscle fibre recordings were performed in well-oxygenated Tyrode's solution with the above drugs to block contraction ($n = 10$ for μ -conotoxin, $n = 12$ for BTS and blebbistatin). Nerve and light evoked potentials were generated as described above. Sharp glass electrodes

(BF100-50-10; World Precision Instruments) with resistances between 20 and 40 M Ω were filled with 3M KCl and connected to an intracellular amplifier (Duo 773 Electrometer, World Precision Instruments). Signals were amplified and bandpass filtered between 1 Hz to 10 kHz and digitized as described above. Recordings were discarded if the initial membrane resting potential (-65 to -90 mV) changed by more than 10% of its original value.

Soleus Muscle Immunofluorescence And Imaging

Soleus muscles were isolated, pinning on cork, embedded in OCT (VWR) and flash frozen in dry ice-cooled isopentane (Sigma). For immunofluorescence, 20 μ m muscle sections were cut on a cryostat (Leica) and mounted on microscope slides. Slides were fixed for 20 min in 4% paraformaldehyde and washed in PBS prior to incubation with antibodies in 0.3% Triton-X (Sigma) in PBS plus 10% goat serum. Incubation with primary antibodies was performed overnight whereas secondary antibody incubation was done over 2 hours. Antibodies consisted of: rabbit anti-GFP IgG (Molecular Probes, 1:1000), mouse anti-dihydropyridine receptor α 2-subunit (Sigma, 1:500) goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen, 1:500) and goat anti-mouse conjugated to Alexa Fluor 546 (Invitrogen, 1:500). Slides were mounted with fluoromount-G (SouthernBiotech). For sarcolemma and T-tubule labeling, soleus muscles were isolated after trans-cardiac perfusion with 4% paraformaldehyde and post fixed for 2 hours at 4°C, subsequently washed in PBS and incubated for one week in a 1 mg/ml Dil solution (Molecular Probes) in PBS at 4°C⁴⁷. Stained muscles were washed with PBS, flash frozen and sectioned as described above. Images were acquired with a Zeiss LSM 710 confocal scanning microscope equipped with Zen 2009 software (Zeiss).

Hindlimb Denervation And Stimulation

Sim1-Ai32 mice were anesthetised with isoflurane and the right sciatic nerve was transected under aseptic conditions. Both proximal and distal ends of the sciatic nerve stump were ligated with 4-0 sutures to prevent regeneration. Hair was removed from the hindlimbs using standard mouse shears. The triceps surae muscles were stimulated transcutaneously using a blue (470 nm) Rebel Star LED positioned immediately above the dorsal shank. The mice were randomized into a stimulated (n = 4) and non-stimulated group (n = 3). The contralateral side served as a non-operated control group. Light pulse stimulation began immediately after the sciatic nerve was transected. The daily stimulation protocol consisted of 5 ms light pulse at 20 Hz for 500 ms every 6 seconds for 1 hour (a total of 600 tetanic contractions per day), a protocol shown to increase muscle force and weight of denervated rat muscle²⁷. At regular intervals, the mice were anaesthetized with isoflurane, the right ankle and knee stabilized and the foot connected to a FT03 force transducer to measure torque at the ankle, generated by a 5 ms light pulse (2.6 mW mm⁻²). After 10 days of daily stimulation, the mice were anaesthetized, the knee firmly fixed in place with a stereotactic frame (Kopf), and the calcaneus was connected to a force transducer to record light activated contractions. Great care was taken not to disturb the blood supply to the tricep surae muscles. After force recordings, the MG muscles were excised and flash frozen as per above. Cross-sections (10 μ m) were cut from the mid region of the muscle before staining with hematoxylin and eosin (Sigma). Whole muscle and individual myofibre cross sectional areas were quantified using Image J (NIH).

Statistical Analysis

Statistics were performed with Prism5 (GraphPad). Student t-tests were done when comparing between only two groups, otherwise one-way ANOVA was performed for multiple groups followed by Dunnett's post-test with p-value significance set at 0.05.

Results

To address these issues, we bred mice expressing Cre recombinase under the control of *Sim1* regulatory sequences with mice harboring a *loxP*-flanked STOP cassette preventing the transcription of a downstream ChR2(H134R)-EYFP fusion gene (known as Ai32 mice). *Sim1* is highly expressed in skeletal muscle early in development¹⁴, while the ChR2/EYFP fusion protein harbors a gain of function mutation (H134R)¹⁵ in the ChR2 protein allowing larger currents^{15,16}, greater light sensitivity and less desensitization^{15,17}.

We first set out to ascertain the cellular localization of the ChR2/EYFP fusion protein in individual myofibres by immunolabelling soleus muscle cross-sections, taken from young *Sim1*-Ai32 mice, for enhanced yellow fluorescent protein (EYFP). Confocal microscopy showed EYFP fluorescence was concentrated at the sarcolemma of each muscle fibre (Figure 1a; left panel). Closer examination showed fainter EYFP immunolabelling of honeycomb structures (Figure 1a; middle panel) resembling T-tubules within individual muscle fibres (Figure 1a; schematic). To ascertain whether ChR2-EYFP was localized to the T-tubule network, we labelled all plasma membrane associated structures with the lipophilic carbocyanine dye 1,1-dioctadecyl-3,3,3,3-tetramethylincarbocyanine perchlorate (DiI)¹⁸. Localization of EYFP and DiI along the sarcolemma and T-tubules was identified in longitudinal sections of soleus myofibres (Figure 1b, middle panel). Further confirmation of EYFP localization to the T-tubules was confirmed with co-localization with dihydropyridine calcium channels (Figure 1b, lower panel). Taken together, these results indicate that ChR2 is distributed throughout structures associated with muscle depolarization and excitation-contraction (EC) coupling.

To ascertain whether muscles with this pattern of ChR2 expression contract when illuminated with light, we anesthetized *Sim1*-Ai32 mice and pulsed the triceps surae muscles through the

skin with blue light generated by a light-emitting diode (LED; 470 nm, 2.6 mW mm⁻², 50 ms light pulse duration) positioned immediately above the dorsal shank musculature (Figure 2a; top left panel). The time-lapse images show a typical example where the illuminated muscles contracted producing an ankle extensor response < 66 ms after the LED was turned on (Supplementary Movie 1). To quantitatively measure contractile responses initiated by light we conducted soleus muscle force recordings *ex vivo* using the same LED and compared those values with forces evoked by neural stimulation using a nerve suction electrode. Twitch forces progressively increased in response to increasing light intensities (Figure 2b, 2b') resulting in values best fitted to a four parameter logistic curve (for 1 ms pulses; $r^2 = 0.991$ with an EC50 = 1.94 mW mm⁻², for 5 ms pulses $r^2 = 0.996$ with an EC50 = 0.529 mW mm⁻²). While greater forces were produced using 5 ms light pulses (Figure 2b''), we found force gradations were better controlled using 1 ms pulses, particularly for values < 20 mN (Figs. 2b, 2b'). Interestingly, a single 1 ms light pulse of 2.6 mW mm⁻² produced similar twitch force as stimulating the nerve with a single electrical pulse (Figure 2b'') indicating that, under these conditions, optical stimulation can be as efficient as neural stimulation. Twitches evoked by 5 ms light pulses, on the other hand, were stronger than those produced by neural stimulation (Figure 2b''). This latter result suggests that contractile force can be graded by varying light pulse duration as well as light intensity. To examine this in more detail, we illuminated the soleus muscle with light pulses (2.6 mW mm⁻²) varying in duration from 0.2 to 1000 ms. Remarkably, not only did contractile responses increase with longer pulse durations, there was no evidence of sag while the light was on (Figure 2c). Twitch force values evoked with light pulses ranging from 0.2 to 10 ms were well fitted to a four parameter logistic curve ($r^2 = 0.99$ with an EC50 = 0.84 ms; Figure 2c') while the forces plateaued with pulses ranging from 10 and 100 ms before increasing further with light pulses exceeding 100 ms (Figure 2c'').

In mammals, contractile force is normally graded by recruiting progressively larger motor units¹⁹ and by modulating their firing frequency²⁰. To ascertain whether stimulating muscles with varying frequencies of light pulses can similarly grade force we illuminated the soleus muscle with 1 and 5 ms light pulses (2.6 mW mm^{-2}) at 5 to 50 Hz for 500 ms. We then compared these values with those evoked by electrical nerve stimulation at the same frequencies. We found that tetanized force can be graded by varying the frequency of optical stimulation and that neural and optical stimulation generated comparable forces (Figure 2d). Taken together, these results show that finely controlled light pulses efficiently produce contractile forces that can be graded by 1) increasing light intensity, 2) changing light pulse duration, and 3) flashing light at varying frequencies.

In order to elucidate how light pulses induce EC coupling in Sim1-Ai32 skeletal muscle fibres we compared contractile forces elicited upon nerve stimulation with those induced by optical stimulation (2.6 mW mm^{-2} , 5 ms pulse) while sequentially applying d-tubocurarine (curare; $10 \mu\text{M}$) and tetrodotoxin (TTX; 500 nM) to soleus muscle/nerve preparations (Figure 3a). As expected, nerve stimulation failed to induce contractions shortly after bath application of curare because of acetylcholine receptors (AChRs) blockade at the NMJs (Figure 3a, 3a'). In contrast, contractions induced by light pulses were not attenuated by curare indicating that ChR2 activation induces EC coupling downstream of synaptic transmission. Interestingly, light pulse induced contractions were reduced by 97% when TTX was added to the bath (Figure 3a & 3a'). These results suggest that light stimulation activates voltage-sensitive sodium channels causing action potentials to be generated within the sarcolemma and/or t-tubules prior to activating the release of Ca^{2+} from the sarcoplasmic reticulum. However, because some force was still produced by light pulses in the presence of TTX (Figure 3a'; 1 mN, or 3% of initial force) these results also indicate that ChR2 activation alone can cause contraction. This likely occurs

because the ChR2 current is either large enough to initiate the release of some Ca^{2+} from the sarcoplasmic reticulum or because Ca^{2+} entered the cell through the channel itself⁹.

To examine electrical properties of optically stimulated muscles (2.6 mW mm^{-2} , 5 ms pulses), we recorded electromyogram (EMG) activity using surface electrodes and compared the magnitude and duration of the signal with those produced by maximum, single pulse nerve stimulation (0.2 ms pulse width). Interestingly, the peak-to-peak amplitude of the EMGs recorded from optically stimulated muscles was significantly less than those measured after nerve stimulation (Figure 3b, 3b'). Because EMG amplitude is proportional to the number of synchronously active muscle fibres^{21,22}, one could conclude that optical stimulation depolarizes fewer myofibres than nerve stimulation. However, we also noticed that the duration of the EMGs measured from optically stimulated muscles was significantly longer than those evoked by nerve stimulation (Figure 3b, 3b"). EMG amplitude wanes if myofibres depolarize asynchronously because the positive voltage from one fibre occurs together with the negative voltage of another²². Thus, in contrast to nerve stimulation, where muscle fibres depolarize relatively synchronously, optical stimulation depolarizes myofibres closest to the light first resulting in asynchronous activation and leading to smaller, but longer lasting EMG signals. In addition, we also noted a second deflection in the EMG when the muscles were light activated with pulses ≥ 5 ms (Figure 3b, asterisk). This second deflection has the same time course as the second depolarization recorded from single muscle fibres during a 5 ms light pulse (Figure 3e). This second depolarization may account for the second deflection in the EMG and, if so, prolongs its duration.

To examine electrical properties of individual myofibres we used intracellular electrodes to record muscle potentials in response to nerve and optical stimulations. To prevent muscle contraction, $1 \mu\text{M}$ μ -conotoxin GIIIB (a muscle specific voltage-gated $\text{Na}_v1.4$ channel blocker)

was added to the perfusion. Under these conditions, nerve stimulation produced large end-plate potentials (EPPs) that were similar in amplitude (~ 18 mV; Figure 3c, 3c' shaded area). In contrast, optical stimulation (5 ms light pulses) produced graded muscle potentials that increased with light intensity (Figure 3c, 3c'). In addition, the rise and fall times of the potentials were significantly slower than nerve-evoked EPPs (Figure 3c). Muscle depolarization could also be graded by increasing the light pulse duration while maintaining light intensity at 1.0 mW mm^{-2} (Figure 3d, 3d'). Under these conditions, maximum depolarization occurred using 10 ms light pulses. Pulses longer than 20 ms or intensities greater than 1.0 mW mm^{-2} caused muscle contractions (data not shown), presumably because the depolarizing current was large enough to activate EC coupling. These persistent depolarizing currents could also account for the sustained contractions observed when muscles were optically stimulated with long light pulses (e.g. Figure 2c). Contraction was blocked by myosin inhibitors, $50 \mu\text{M}$ BTS (N-benzyl-p-toluene sulphonamide)²³ and $10 \mu\text{M}$ blebbistatin²⁴⁻²⁶, applied simultaneously to the perfusion prior to intracellular potential recording of neural and optical stimulations (Figure 3e). Optical stimulation pulses longer than 1 ms consistently generated an action potential of identical amplitude to the neural-induced action potential (Figure 3e'). As expected from the slower kinetics of ChR2, the optically generated action potentials were 4 ± 1 ms delayed after the neural-induced action potentials. A second smaller depolarization was seen with stimuli greater than 1 ms and was followed by a depolarization plateau with pulses ≥ 20 ms duration until the light would be turned off (Figure 3e''). Taking together, these results show that optical stimulation depolarizes myofibres asynchronously, induces EC coupling downstream of AChR activation, can generate a second depolarization following the initial action potential and can maintain prolonged tetanic contractions by sustaining persistent muscle depolarizing currents.

To investigate whether channelrhodopsin expression in muscle could be used to restore function to permanently denervated muscles, we cut and ligated the sciatic nerve in Sim1-Ai32 mice. One cohort of animals (designated stimulated) received daily transcutaneous light stimulation (5 ms pulses at 20 Hz for 500 ms every 6 sec for one hour) to the triceps surae muscles for 10 days, starting immediately after the nerve was transected²⁷. Another cohort was not stimulated with light (designated non-stimulated). EMG recordings from denervated medial gastrocnemius (MG) muscles in freely moving Sim1-A132 mice showed no signs of muscle activation indicating that ambient light did not cause muscle contractions (data not shown). The contralateral muscles served as unoperated controls. Mice were anesthetized at regular intervals, starting immediately before nerve transection, in order to measure twitch force produced at the ankle joint upon transcutaneous light stimulation (2.6 mW mm⁻² with a 5 ms pulse width). Figure 4a shows average daily forces produced by the optically stimulated and non-stimulated denervated muscles, recorded over a 10-day period. In both cases, force produced at the ankle by the triceps surae muscles decreased significantly from pre-operative levels. However, the attenuation in force was significantly less for muscles receiving daily optical stimulation such that the force of optically stimulated muscles plateaued at ~70% pre-operative levels while the non-stimulated muscles plateaued at ~40% (Figure 4a, 4b). Ten days after nerve transection the MG muscles were isolated *ex vivo* and their tendons attached to a force transducer. Light-activated contractions generated from the optically stimulated and non-stimulated muscles were then compared to the contractile forces generated by optically stimulating the contralateral control MG muscle. Figure 4c shows that, while the contractile force of MG muscles receiving daily optical-stimulation was significantly less than control values (~70%), they were also significantly stronger than non-stimulated muscles. This difference in force was reflected in their wet weights (Figure 4d) and overall size (Figure 4e). The cross-sectional areas of the optically stimulated muscles were significantly smaller than control

muscles, but significantly larger than non-stimulated muscles (Figure 4f). As expected, non-stimulated denervated MG myofibres were also significantly smaller than fibres in control and optically stimulated muscles (Figure 4g, 4h) indicating that denervation atrophy was most pronounced in this experimental group. Taken together, these results indicate that daily transcutaneous light stimulation can significantly decrease the effects of denervation in muscles expressing ChR2.

Discussion

In this study, we show that ChR2 is abundantly expressed within the sarcolemma and T-tubules of skeletal muscle fibres in Sim1-Ai32 mice. This expression pattern allows for efficient control of muscle contraction using light pulses from a 470 nm LED, where maximal force obtained by optical stimulation was comparable to direct nerve stimulation. Furthermore, muscle force could be graded in a controlled manner by increasing light pulse intensity, duration or by modulating their frequency. Muscle fibres depolarized in a graded manner relative to light stimulation until reaching action potential threshold and for longer pulse durations remained depolarized for the duration of the light pulse. Although the gradation in force was likely due to an increase in the number of muscle fibres activated and the contractile strength of each fibre, it is not the same as neurally evoked activation in force, which normally occurs according to the size principle¹⁹.

Finally we found that muscle fibre atrophy was significantly attenuated when the denervated muscles were optically stimulated through the skin for one hour a day. This decrease in atrophy resulted in significantly greater contractile forces compared to chronically denervated muscles. Together, this proof of principle study shows that stimulation of ChR2 expressing myofibres with light can restore function to denervated muscles and reduce pathophysiological changes associated with injury. Furthermore, it extends on recent work by Sasse and colleagues²⁸, who showed optical control of cardiac muscle, as well as the reports by Delp and colleagues illustrating muscle activation via optical stimulation of sciatic nerve²⁹.

To date, restoration of function to paralyzed muscles typically involves electrical stimulation. FES has been studied and used to restore function to upper and lower extremities, the diaphragm, bladder and bowels³. Electrical impulses are typically applied to electrodes near, or around, the peripheral nerve causing motor axon depolarization and muscle contraction. This approach, however, is not useful if the target muscles are completely denervated, as occurs

after SCIs^{30,31}, motor neuron diseases such as ALS³², and severe peripheral nerve injuries³³, such as occurs after a brachial plexus avulsion. It is not useful because denervated muscles require substantially more current to induce contractions compared to neural stimulation. High currents promote a multitude of problems including electrode corrosion, tissue damage, discomfort and unwanted stimulus spread to neighboring muscles³⁴.

To circumvent these issues, we^{6,7} and others^{5,8}, restored innervation and function to denervated muscles by transplanting embryonic motor neurons, or motor neurons derived from pluripotent cells, into the peripheral nerve near the nerve-muscle entry point. Electrically stimulating the transplanted neurons generated between 12-40% of the original contractile force. While promising, this approach has many technical and biological obstacles that may ultimately circumvent its use clinically. Foremost among these is the justifiable concern that teratoma will form³⁵. Although the risk of teratoma formation can be reduced experimentally by removing unwanted pluripotent cells prior to transplantation using small molecules³⁶, immunodepletion³⁷, genetic selection³⁸, or by introducing a cytotoxic antibody³⁹, in practical terms it may be very difficult to guarantee their complete absence to a regulatory agency. Furthermore, it is uncertain whether progenitor or partially differentiated cells remaining in the transplant will someday become a health risk to the transplant recipient during their life span⁴⁰.

Transplantation of motor neurons to restore muscle function after injury will likely occur only after endogenous recovery is complete, a process that can take several months in humans⁴¹. This reality further obstructs the use of motor neuron transplantation because motor axon regeneration and muscle fibre reinnervation is very poor when axotomy and/or muscle denervation is prolonged^{11,12,41-43}. Thus, while restorative transplantation therapies using motor neurons derived from pluripotent cells hold promise, practical limitations may ultimately hinder clinical application of this technology.

Restoration of muscle function by direct optical activation of myofibres abates risks of teratoma formation and bypasses the need to restore function by regenerating axons through an inhospitable environment. Skeletal muscles are amenable to different delivery mechanisms for gene delivery such as viral transduction. Indeed, gene transfer into adult muscle has recently been shown to express the ChR2/H134R resulting in functional muscle contraction under light stimulation⁴⁴. While our study is largely a proof of principle using transgenic animals, it is possible that, in combination with gene transfer technology, this strategy may become clinically useful for restoring function to specific muscles permanently denervated by injury or disease. However, the success of this strategy will be highly dependent on viral delivery method, sustained expression of ChR2 in infected fibres and kinetics of engineered ChR2. Nonetheless, restoring even a small amount of function will improve independence and have a profoundly positive effect on the adverse psychological, social, and economic factors associated with denervation injuries, ranging from peripheral nerve injuries³³ to SCIs¹.

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Ethics

The Dalhousie Animal Use Committee, in accordance with the standards of the Canadian Council of Animal Care, approved all procedures.

Author Contributions

PM, BS, YZ and VFR conceived and designed experiments. PM and BS performed experiments and data analysis. PM, BS, YZ and VFR wrote the manuscript.

Conflicts Of Interest

No conflict of interest to declare.

References

1. Anderson, K. D. Targeting recovery: priorities of the spinal cord-injured population. *J Neurotrauma* **21**, 1371–1383 (2004).
2. Prochazka, A., Mushahwar, V. K. & McCreery, D. B. Neural prostheses. *J Physiol (Lond)* **533**, 99–109 (2001).
3. Popović, D. B. Advances in functional electrical stimulation (FES). *Journal of Electromyography and Kinesiology* **24**, 795–802 (2014).
4. Thomas, C. K., Zaidner, E. Y., Calancie, B., Broton, J. G. & Bigland-Ritchie, B. R. Muscle Weakness, Paralysis, and Atrophy after Human Cervical Spinal Cord Injury. *Exp Neurol* **148**, 414–423 (1997).
5. Thomas, C. K., Erb, D. E., Grumbles, R. M. & Bunge, R. P. Embryonic cord transplants in peripheral nerve restore skeletal muscle function. *J Neurophysiol* **84**, 591–595 (2000).
6. Yohn, D. C., Miles, G. B., Rafuse, V. F. & Brownstone, R. M. Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy. *Journal of Neuroscience* **28**, 12409–12418 (2008).
7. Toma, J. S. *et al.* Motoneurons derived from induced pluripotent stem cells develop mature phenotypes typical of endogenous spinal motoneurons. *Journal of Neuroscience* **35**, 1291–1306 (2015).
8. Bryson, J. B. *et al.* Optical Control of Muscle Function by Transplantation of Stem Cell-Derived Motor Neurons in Mice. *Science* **344**, 94–97 (2014).
9. Nagel, G. *et al.* Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc Natl Acad Sci USA* **100**, 13940–13945 (2003).
10. Knoepfler, P. S. Deconstructing Stem Cell Tumorigenicity: A Roadmap to Safe Regenerative Medicine. *Stem Cells* **27**, 1050–1056 (2009).
11. Fu, S. Y. & Gordon, T. Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy. *J Neurosci* **15**, 3876–3885 (1995).
12. Fu, S. Y. & Gordon, T. Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. *J Neurosci* **15**, 3886–3895 (1995).
13. Gordon, T. & Fu, S. Y. Long-term response to nerve injury. *Adv Neurol* **72**, 185–199 (1997).
14. Coumailleau, P. & Duprez, D. Sim1 and Sim2 expression during chick and mouse limb development. *International Journal of Developmental ...* (2009).

15. Nagel, G. *et al.* Light Activation of Channelrhodopsin-2 in Excitable Cells of *Caenorhabditis elegans* Triggers Rapid Behavioral Responses. *Current Biology* **15**, 2279–2284 (2005).
16. Madisen, L. *et al.* A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. *Nat Neurosci* **15**, 793–802 (2012).
17. Lin, J. Y., Lin, M. Z., Steinbach, P. & Tsien, R. Y. Characterization of engineered channelrhodopsin variants with improved properties and kinetics. *Biophysical Journal* **96**, 1803–1814 (2009).
18. O'Connell, K., Whitesell, J. D. & al, E. Localization and mobility of the delayed-rectifier K⁺ channel Kv2. 1 in adult cardiomyocytes. *American Journal of ...* (2008).
19. Henneman, E. & Somjen, G. Functional significance of cell size in spinal motoneurons. *Journal of ...* (1965).
20. Milner Brown, H. S., Stein, R. B. & Yemm, R. Changes in firing rate of human motor units during linearly changing voluntary contractions. *J Physiol (Lond)* **230**, 371–390 (1973).
21. Milner Brown, H. S. & Stein, R. B. The relation between the surface electromyogram and muscular force. *J Physiol (Lond)* **246**, 549–569 (1975).
22. de Zepetnek, J. E. T., Gordon, T., Stein, R. B. & Zung, H. V. Comparison of force and EMG measures in normal and reinnervated tibialis anterior muscles of the rat. *Can. J. Physiol. Pharmacol.* **69**, 1774–1783 (2011).
23. Macdonald, W. A., Pedersen, T. H., Clausen, T. & Nielsen, O. B. N-Benzyl-p-toluene sulphonamide allows the recording of trains of intracellular action potentials from nerve-stimulated intact fast-twitch skeletal muscle of the rat. *Experimental Physiology* **90**, 815–825 (2005).
24. Straight, A. F. *et al.* Dissecting Temporal and Spatial Control of Cytokinesis with a Myosin II Inhibitor. *Science* **299**, 1743–1747 (2003).
25. Limouze, J., Straight, A. F., Mitchison, T. & Sellers, J. R. Specificity of blebbistatin, an inhibitor of myosin II. *J Muscle Res Cell Motil* **25**, 337–341 (2004).
26. Fedorov, V. V. *et al.* Application of blebbistatin as an excitation–contraction uncoupler for electrophysiologic study of rat and rabbit hearts. *Heart Rhythm* **4**, 619–626 (2007).
27. Willand, M. P. *et al.* A New System and Paradigm for Chronic Stimulation of Denervated Rat Muscle. *J Med Biol Eng* **31**, 87–92 (2011).
28. Bruegmann, T. *et al.* Optogenetic control of heart muscle in vitro and in vivo. *Nat Meth* **7**, 897–900 (2010).
29. Llewellyn, M. E., Thompson, K. R., Deisseroth, K. & Delp, S. L. Orderly recruitment of motor units under optical control in vivo. *Nat Med* **16**, 1161–1165 (2010).

30. Yang, J. F., Stein, R. B., Jhamandas, J. & Gordon, T. Motor unit numbers and contractile properties after spinal cord injury. *Ann Neurol* **28**, 496–502 (1990).
31. Thomas, C. K., Sesodia, S., Erb, D. E. & Grumbles, R. M. Properties of medial gastrocnemius motor units and muscle fibers reinnervated by embryonic ventral spinal cord cells. *Exp Neurol* **180**, 25–31 (2003).
32. Dantes, M. & McComas, A. The extent and time course of motoneuron involvement in amyotrophic lateral sclerosis. *Muscle Nerve* **14**, 416–421 (1991).
33. Choi, P. D., Novak, C. B., Mackinnon, S. E. & Kline, D. G. Quality of life and functional outcome following brachial plexus injury. *J Hand Surg Am* **22**, 605–612 (1997).
34. Plenk, H. The role of materials biocompatibility for functional electrical stimulation applications. *Artificial organs* **35**, 237–241 (2011).
35. Lee, A. S., Tang, C., Rao, M. S., Weissman, I. L. & Wu, J. C. Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies. *Nat Med* **19**, 998–1004 (2013).
36. Lee, M.-O. *et al.* Inhibition of pluripotent stem cell-derived teratoma formation by small molecules. *Proc Natl Acad Sci USA* **110**, E3281–90 (2013).
37. Tang, C. *et al.* An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. *Nat Biotechnol* **29**, 829–834 (2011).
38. Chung, S. *et al.* Genetic selection of sox1GFP-expressing neural precursors removes residual tumorigenic pluripotent stem cells and attenuates tumor formation after transplantation. *J Neurochem* **97**, 1467–1480 (2006).
39. Choo, A. B. *et al.* Selection against undifferentiated human embryonic stem cells by a cytotoxic antibody recognizing podocalyxin-like protein-1. *Stem Cells* **26**, 1454–1463 (2008).
40. Cunningham, J. J., Ulbright, T. M., Pera, M. F. & Looijenga, L. H. J. Lessons from human teratomas to guide development of safe stem cell therapies. *Nat Biotechnol* **30**, 849–857 (2012).
41. Calancie, B., Molano, M. R. & Broton, J. G. EMG for assessing the recovery of voluntary movement after acute spinal cord injury in man. *CLINICAL NEUROPHYSIOLOGY* **115**, 1748–1759 (2004).
42. Gutmann, E. & Young, J. Z. The re-innervation of muscle after various periods of atrophy. *J Anat* **78**, 15–43 (1944).
43. Gutmann, E. Effect of delay of innervation on recovery of muscle after nerve lesions. *J Neurophysiol* **11**, 279–294 (1948).
44. van Bremen, T. *et al.* Optogenetic control of contractile function in skeletal muscle. *Nat Comms* **6**, 1–8 (2015).

45. Zhang, Y. *et al.* V3 Spinal Neurons Establish a Robust and Balanced Locomotor Rhythm during Walking. *Neuron* **60**, 84–96 (2008).
46. Gosgnach, S. *et al.* V1 spinal neurons regulate the speed of vertebrate locomotor outputs. *Nature* **440**, 215–219 (2006).
47. Lukas, J.-R. *et al.* Carbocyanine Postmortem Neuronal Tracing: Influence of Different Parameters on Tracing Distance and Combination with Immunocytochemistry.

Figure Legends

Figure 1 – Chr2 Expression In Sim1-Ai32 Mouse Muscle Fibres

(a) Confocal image shows ChR2/YFP fusion protein is highly expressed in sarcolemma (left panel, scale $20\mu\text{m}$) and lightly expressed in honeycomb structures within individual myofibres (middle panel, scale $5\mu\text{m}$). Illustration (right panel) shows key anatomical structures associated with myofibres including the sarcolemma, myofibrils and T-tubules. (b) Confocal image of a Sim1-Ai32 muscle fibre longitudinal section labelled with Dil (top panel, scale $10\mu\text{m}$) or DHP calcium channels (bottom panel, scale $10\mu\text{m}$) to show all membrane associated structures including T-tubules. Dil and DHP calcium channel staining both co-localize with the EYFP/ChR2 fusion protein indicating that ChR2 is expressed in the sarcolemma and T-tubule network.

Figure 2 – Optical Stimulation Can Modulate Force By Varying Light Intensity, Light Duration And Light Pulse Frequency

(a) Hindlimb muscle contraction induced by blue light emitted from an LED positioned 1 cm away. Images represent five serial frames, 33, 66, 99 and 132 ms after the onset of a 50 ms light pulse. (b) Example of force gradations obtained by incrementally increasing light intensity using a 1 or 5 ms pulse of light. (b') Quantification of force at different light intensities using a 1 or 5 ms pulse of light (2.6 mW mm^{-2}). (b'') A 1 ms light pulse, at 2.6 mW mm^{-2} , produces the same force as nerve evoked twitch contraction while a 5 ms pulse at the same light intensity produces significantly more force. (c) Force profiles generated while incrementally increasing light pulse duration from 0.2 ms to 1 second. (c') Quantification of force generated using light pulses (2.6 mW mm^{-2}) varying in duration from 0.2 to 10 ms. (c'') Quantification of force generated using light pulses (2.6 mW mm^{-2}) varying in duration from 0.2 to 1000 ms. Note that

stimulus duration is plotted on a logarithmic scale. (d) Tetanized force can be graded by varying the frequency of neural or optical stimulation. Bar graphs expressed as mean \pm SD. ** $p < 0.01$

Figure 3 – Muscle Properties During Light Induced Contractions

(a) Sequential bath application of curare (10 μ M) and TTX (500 nM) to soleus muscles shows that nerve evoked contractions were blocked by curare while contractions induced by 5 ms light pulses were not. TTX, on the other hand, blocked light evoked contractions. There was partial recover of light evoked force after a 20 minutes wash out. (a') Quantification of nerve and light evoked force, before and after bath application of curare and TTX. While light evoked contractions were dramatically attenuated in the presence of TTX, they remained minimally present despite TTX. (b) Examples of EMGs evoked through nerve stimulation (gray trace) or with a 2.6 mW mm^{-2} , 5 ms light pulse (black trace). The asterisk represents a second depolarization after light stimulation. (b') Quantification of EMG amplitude and duration (b'') using nerve stimulation or a 2.6 mW mm^{-2} , 5 ms light pulse. Note that light stimulation produces EMGs that are smaller in amplitude, but longer in duration, compared to nerve stimulation. (c) Examples of muscle potentials recorded from soleus myofibres using nerve stimulation (gray trace) or by increasing the light intensity (5 ms pulse, black traces). (c') Quantification of EPP amplitude at increasing optically intensities. Shaded area shows the range in EPPs recorded at the NMJ upon nerve stimulation. (d) Examples of muscle potentials recorded from myofibres using light pulses of varying lengths (1.0 mW mm^{-2} light intensity). (d') Quantification of light evoked muscle potentials recorded at varying light pulse durations. (e) Quantification of light evoked muscle potentials under myosin inhibitors with varying pulse durations. Arrows represent stimulus artifact from LED turning on and off which merges with the depolarization at 5 and 10 ms. Optically evoked depolarizations compared to neural-evoked potentials. Bars and symbols are expressed as mean \pm SD. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$

Figure 4 – Daily, Transcutaneous Light Stimulation Attenuates Denervation Atrophy Of Completely Denervated Muscles And Improved Contractile Force

(a) Average forces produced by optically stimulated and non-stimulated, denervated muscles recorded over 10 days following sciatic nerve transection. (b) Average twitch force recorded from contralateral (control), non-stimulated and optically stimulated triceps surae muscles 10 days after nerve transection. (c) Average light induced twitch force and (d) weight wet of control, non-stimulated and optically stimulated MG muscles 10 days after nerve transection. (e) Whole muscle cross-sections of control, non-stimulated and optically stimulated MG muscles stained for H&E. (f) Mean muscle cross-sectional areas of the three muscle groups. (g) Frequency histograms comparing the cross-sectional area of myofibres in control, non-stimulated, and optically stimulated MG muscles. (h) Cumulative frequency showing the shift to smaller muscle fibres in the non-stimulated muscles compared to optically stimulated and control muscles.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar in e = 1 mm.

Supplemental Movie 1 – Transcutaneous Optical Activation Of Muscle Contraction

See appendix one.

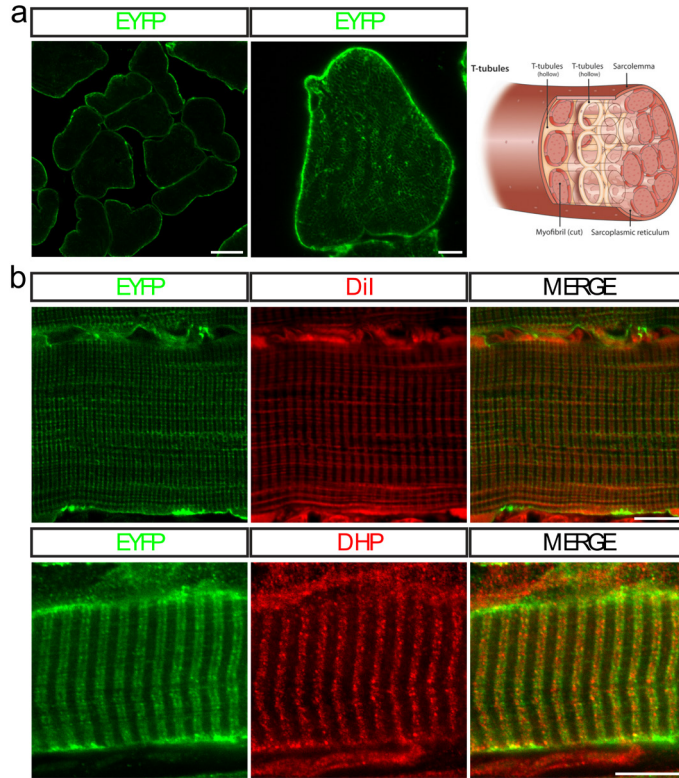


Figure 1 – ChR2 expression in Sim1-Ai32 mouse muscle fibres

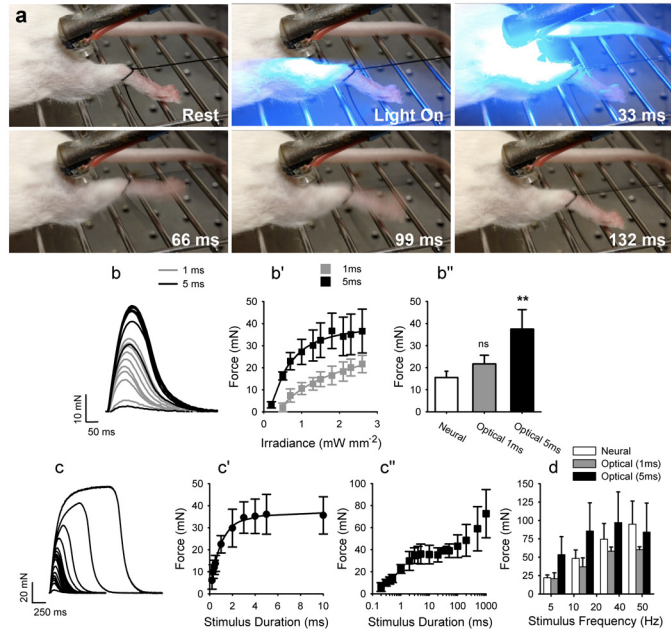


Figure 2 – Optical stimulation can modulate force by varying light intensity, light duration and light pulse frequency

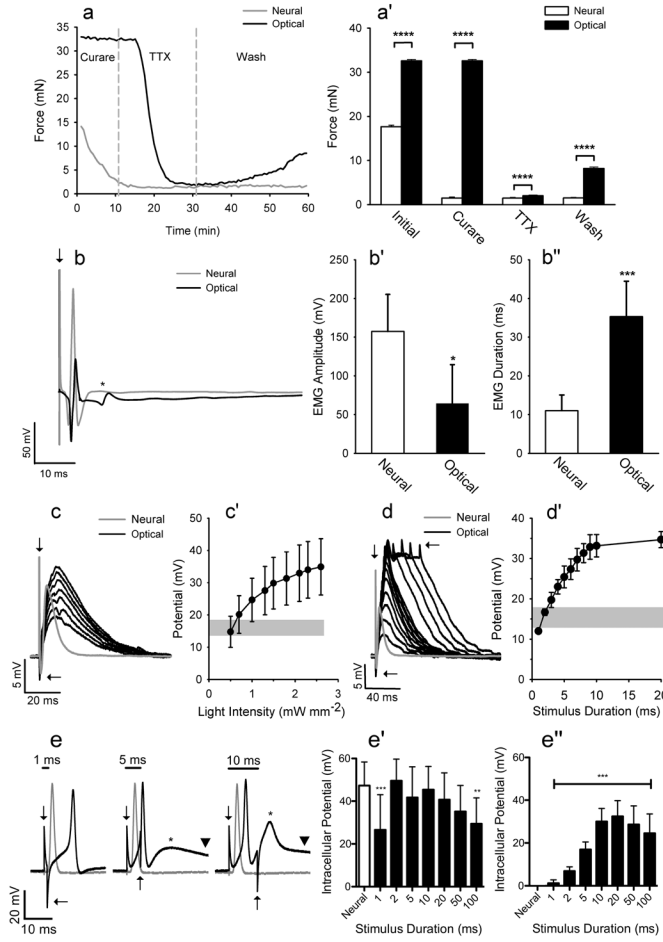


Figure 3 – Muscle properties during light induced contractions

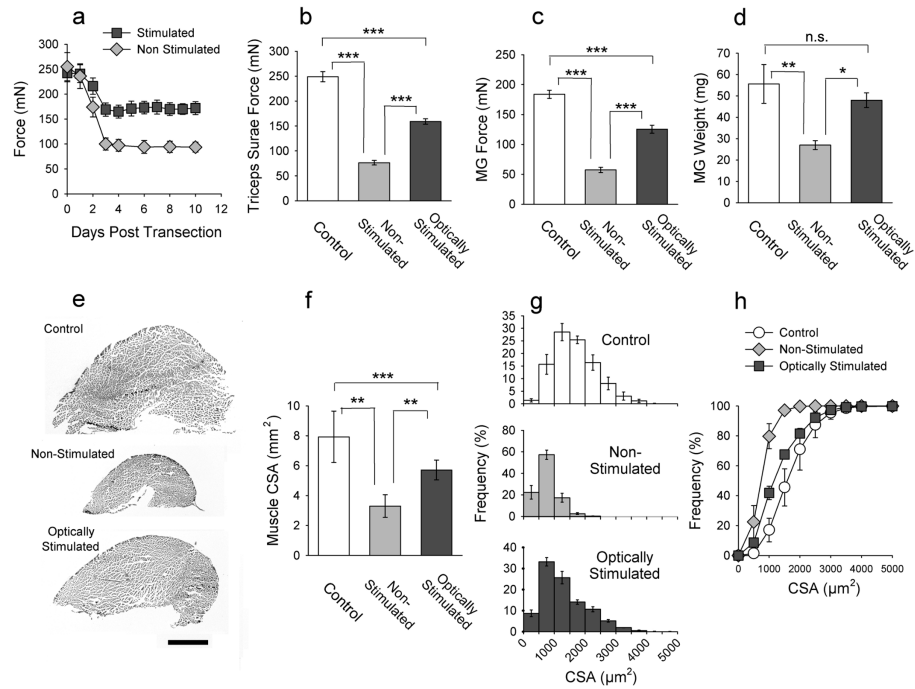


Figure 4 – Daily, transcutaneous light stimulation attenuates denervation atrophy of completely denervated muscles and improved contractile force

Chapter Four: Spontaneous Circuit Activity Of Transplanted Embryonic Stem Cell-Derived Motoneurons

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Abstract

During the formation of spinal cord locomotor circuit, embryonic neurons are spontaneously active. Early on, this spontaneous activity is dependent on cholinergic transmission possibly originating from motoneurons. Motoneurons can be generated from embryonic stem cells and transplanted in the tibial nerve to study innervation properties outside of the spinal cord. Here, we studied the spontaneous neuronal activity occurring from transplanted embryonic stem cell derived motoneurons disconnected from the spinal cord. Embryonic stem cell-derived motoneurons were transplanted into the tibial nerve after denervation by nerve transection. Three months later, the medial gastrocnemius force was studied *ex vivo*. Spontaneous and evoked muscle contractions were subjected to neurotransmitter blockers to elucidate the network connections. Half of transplants spontaneously generated rhythmic muscle contractions. The generated activity was variable between transplants; some demonstrating spontaneous contractions while others had burst of contractions only if electrically evoked. Activity was dependent on glutamate transmission, and was modulated by GABAergic, glycinergic and muscarinic cholinergic inputs demonstrating the presence of interneurons involved in this self-assembled circuit. Self-assembly of a spontaneously active circuit from transplanted embryonic stem cell-derived motoneurons emphasizes the intrinsic nature of neurons to form synaptic connections in order to integrate within a neuronal network. Spontaneous activity may be essential for the proper development of embryonic motoneurons and may be unavoidable. These findings demonstrate the necessity to further our understanding of neuronal subtypes generated from stem cell neural differentiation and the influent factors affecting synaptogenesis among transplanted neurons.

Introduction

Spontaneous activity of neurons during embryogenesis is important for the development of complex neural circuits (Kirkby et al., 2013). Such activity plays a role in synapse development as well as axon path-finding (Gomez and Spitzer, 1999; Hanson and Landmesser, 2004). In the spinal cord, release of acetylcholine from developing MNs has been shown to be crucial for the development of locomotor networks early in embryogenesis, as mutants lacking the ability to synthesise this transmitter in MNs develop abnormal coordination associated with long bursts (Myers et al., 2005). This is a transient requirement, as later in development eliminating cholinergic neurotransmission has little effect (Myers et al., 2005) and interneuronal glutamatergic and glycine/GABA transmission play increasing roles in the bursting behaviour (Rosato-Siri et al., 2004). Thus through development, various neuronal populations and various transmitter phenotypes play different roles in spontaneous bursting activity at different time points in development, and this activity is essential for the development of circuits.

Motoneurons can be derived from embryonic stem cells through exogenous application of signalling factors present in the ventral spinal cord during development (Wichterle et al., 2002). Although this results in enrichment of MNs in these cultures (Wichterle et al., 2002; Miles et al., 2004), other neuronal types also develop (Wichterle et al., 2002; Deshpande et al., 2006). Some of these neurons express markers associated with either excitatory or inhibitory ventral spinal interneuronal types (Deshpande et al., 2006), such as V0 and V2 interneurons. Given that primary neuronal cultures have been shown to be spontaneously active and self-assemble into active networks *in vitro* (Streit et al., 2001; Van Pelt et al., 2004; Arnold et al., 2005), it may be that these ES cell derived neurons could also do so. Knowledge of whether these other types of neurons are active in these cultures and whether they form microcircuits will lead to an

understanding of spinal circuit formation and potentially the role of such circuits in transplant efficacy (Yohn et al., 2008).

Following transplantation of ESCMNs into transected nerves of mice, MNs survive and functionally innervate muscle (Yohn et al., 2008). Given the importance of spontaneous activity in normal development, we asked whether there is evidence of network formation and spontaneous activity in transplanted ESCMN. We therefore studied spontaneous network activity in these transplants, which are isolated from the central nervous system. We found spontaneous and evoked rhythmic activity causing rhythmic muscle contractions. This activity was glutamate-dependent, suggesting formation of circuits with excitatory interneurons. Circuit function was modulated by acetylcholine and GABA/glycine. Thus, self-organized circuitry forms after transplantation and is capable of driving rhythmic muscle contraction.

Methods

Embryonic Stem Cell Derived Motoneurons

Generation of embryonic stem cell derived MNs has been previously described (Wichterle et al., 2002; Miles et al., 2004; Yohn et al., 2008). In summary, HBGB6 stem cells expression GFP under the motoneuronal promoter Hb9 were maintained as undifferentiated colonies over mitomycin-treated primary mouse embryonic fibroblasts in stem cell media. Stem cells were agglomerated as embryonic bodies before differentiation with smoothen agonist (500 nM, Enzo) and retinoic acid (1 μ M, Sigma) for 5 days. The presence of MNs was confirmed by the expression of GFP and on average composed 20% of the cell population.

ESCMN Transplantation

All procedures were performed in accordance with protocols approved by the Dalhousie University Animal Care Committee, and conformed to the standards of the Canadian Council of Animal Care. Details of the ESCMN dissociation and transplantation can be found in a previous publication (Yohn et al., 2008; Magown et al., pending). In summary, embryonic bodies were treated with 1 μ g/ml mitomycin C (except for immediate transplants) for 2 hours and washed prior to being enzymatically dissociated with Tryple-express (Gibco) with 0.01% (w/v) DNaseI (Sigma-Aldrich). The cell suspension was resuspended at 10^6 cells per 10 μ L of DFK10 with 10 μ g/ml GDNF (Milipore), 20 μ g/ml CNTF (Chemicon) and 0.01% DNaseI (Sigma-Aldrich).

Transplantation was performed in 5 week-old mice either immediately after nerve transection or after a delay of 1, 2 or 4 weeks post transection as previously described (Magown et al., pending). Briefly, the tibial nerve was exposed via a lateral thigh approach and transected proximal to the branching of the nerve to the medial gastrocnemius (MG). The proximal tibial

nerve stump was ligated and buried into the adjacent muscle to prevent reinnervation. Ten thousand cells in 0.1 μL were transplanted in the distal tibial nerve with a glass pipette.

***In Vitro* Electrophysiological Recordings**

The MG muscle and the transplanted tibial nerve were harvested 3 months post transplantation and maintained in an *in vitro* chamber circulating oxygenated mouse Tyrode at room temperature (Yohn et al., 2008). Stimulation to evoke bursting activity was provided to the MG nerve with a polyethelene suction electrode via a square pulse stimulator (S88, Grass Technologies) and a stimulus isolation unit (PSIU6, Grass Technologies). Stimulation was 1.5x the maximal stimulus threshold (usually around 10 V, 100 μA) with a duration of 0.2 ms, provided as either 3 pulses at 5 Hz or 25 pulses at 50 Hz. The minimal number of pulses to elicit bursting activity was used. Forces were measured with a force transducer (FT03, Grass Technologies) connected to an AC/DC strain gage amplifier (P122, Grass Technologies). Signals were recorded via a Digidata 1320A, using Axoscope 9.2 software (Molecular Devices).

The following drugs were used: CNQX 10 μM (disodium salt hydrate, #115066-14-3, Sigma), APV 100 μM (#76326-31-3, Sigma), bicuculline 10 μM (#485-49-4, Sigma), strychnine hydrochloride 1 μM (#1421-86-9, Sigma), atropine 10 μM (51-58-8, Sigma), NMDA 5 μM (#6384-92-5, Sigma), serotonin hydrochloride (5-HT) 10 μM (#153-98-0, Sigma) and dopamine hydrochloride 50 μM (#62-31-7, Sigma). All drugs were added as a concentrated stock to the circulating Tyrode solution to give the final concentrations indicated.

Statistical Analysis

Statistical analysis was performed before and after drug infusion on each animal individually. Because of the high variability of responses between animals, results were not combined for analysis. A false discovery rate (Q) of 1% was considered as the limit for discovery. For each

animal, effects from infused drug compared to baseline were tested for significance with an unpaired t-test with Mann-Whitney correction. Bar graphs represent means \pm standard deviations. Statistics were performed with Prism6 (GraphPad).

Results

Motoneurons derived from embryonic stem cells were transplanted into the tibial nerve either acutely after transection or after a denervation period of up to four weeks and MG forces were recorded *ex vivo* three months after transplantation (Yohn et al., 2008). Out of 24 transplanted mice (the same mice as reported in Magown et al., pending), 17 demonstrated contraction of the MG upon electrical stimulation of the transplant site, indicating functional engraftment. Of these 17 mice, nine (53%) had rhythmic contractions, six of which were spontaneously rhythmic in the absence of electrical stimulation (Movie 1), and three of which had bursts of contractions evoked by either a single electrical pulse or a short train of pulses (Figure 1A). Cutting the tibial nerve distal to the transplant resulted in complete ablation of rhythmic contractions in all 9 mice. Therefore, transplantation of ESCMNs led to spontaneous or evoked rhythmic muscle contractions.

We next investigated the role of glutamatergic transmission in the spontaneous contractions. The addition of the glutamate receptor blockers, CNQX and APV to the preparations with evoked bursting prevented further stimulus-evoked bursting ($n = 2$; Figure 1A). In transplants with spontaneous activity, glutamate blockade significantly reduced the force amplitude (to 28% and 63% of baseline, $p < 0.05$) and doubled the mean instantaneous frequency of contractions ($n = 2$; Figure 1B, C). This suggests that fewer MNs were active, and that activity between individual MNs was less coordinated. Autocorrelation following glutamate blockade revealed a fast rhythm but of low correlation (Figure 1D). Force histogram showed a range of forces with very few small events before the application of glutamate transmission blockade (Figure 1E). These data indicate that blocking glutamatergic transmission disrupted an intrinsic circuit, resulting in a reduction of motor output, incoordination of MN firing and an increase in spontaneous event frequency. Thus, glutamatergic inputs were driving ESCMNs activity.

We next asked whether GABAergic and glycinergic inputs to ESCMNs played a role in rhythmicity. In one of two transplants that were spontaneously active, there was a transient response, with an increase in force (Figure 1F-H). After 30 minutes without washout, forces returned to baseline (denoted “late” on Figure 1G). No further effect was seen on washout. While there was no change in frequency of the bursts, the activity became more organized over time, as demonstrated by autocorrelation analysis (Figure 1I). Thus, GABA/glycine neurotransmission, which may have contributed to the amplitudes of the burst, led to desynchronization of MN activity.

We next focused on the effect of cholinergic transmission, given the known role of cholinergic activity in the generation of spontaneous activity in embryonic spinal cords (Wenner and O'donovan, 2001; Myers et al., 2005; Czarnecki et al., 2014; Gonzalez-Islas et al., 2015). As nicotinic blockade would block muscle contraction, we studied muscarinic responses ($n = 3$). Addition of atropine resulted in a prolongation of the episodes of stimulus-evoked bursting (Figure 2A, B, $n = 1$). Event amplitudes progressively increased towards the end of the burst. In transplants with spontaneous activity ($n = 2$), the time interval between episodes decreased after atropine application (Figure 2E all events). Furthermore, a background activity of low amplitude events (2.7 ± 0.5 mN at 2.8 Hz) could be identified among large amplitude events (17.8 ± 6.6 mN at 0.05 Hz; Figure 2C, D). Large amplitude events were thus defined as those with a force greater than 6 mN whereas small events had forces lower than 6 mN. While atropine had no effect on the overall forces produced (of either small or large amplitude events, Figure 2G), the inter-event intervals of small events increased while those of large events decreased (Figure 2E). The frequency histogram of event forces demonstrates that the majority of events were of small amplitude but these decreased in frequency after atropine to be

replaced by larger events (Figure 2H). Muscarinic blockade did not increase the event amplitude above twitch force (24.36 mN), which would have required summation of contractions. These findings are similar to the effects of atropine on spontaneous activity of cortical neurons in culture (Hammond et al., 2013), and indicate that in our transplant-generated networks, blocking muscarinic receptors resulted in an increased number of episodes, a slowing of spontaneous activity within each episode, and a synchronization of firing among MNs.

As the small amplitude events likely represent desynchronized MN activity, we next focused on the effects of muscarinic receptors on these events in isolation. To do so, we added atropine after blocking glutamate (and GABA/glycine) transmission. Following atropine application, residual small amplitude muscle contractions remained, but there was a slowing of event frequency (Figure 2I-K) without effect on force amplitude. Taken together, these results suggest that muscarinic receptors contribute to MN excitability, and this may have modulated the overall bursting activity seen. Whether the source of the acetylcholine was motor axon collaterals or cholinergic interneurons (Miles et al., 2004) is not clear.

Given the above evidence of circuit formation, we asked whether transplanted ESCMNs could sustain rhythmic contractions by adding the neurochemicals that induce locomotor-like rhythmicity in the mouse spinal cord: NMDA, 5-HT and DA (Jiang et al., 1999). The addition of these neurochemicals did not transform the evoked bursting activity ($n = 2$) or spontaneous bursting activity ($n = 2$) into sustained activity. However, evoked bursting episodes were prolonged (Figure 3A, B) and in spontaneously bursting transplants, contraction forces, instantaneous frequency and episode frequency were increased (Figure 3D-G). Autocorrelation shows an oscillation of low amplitude under NMDA, 5-HT, and DA (Figure 3C) suggesting that the neurotransmitters excited the neuronal network and not only the MNs. That is, addition of

NMDA, 5-HT, and DA resulted in an increase in rhythmic motor output, suggesting the possibility that elements of a locomotor “central pattern generator” had formed.

Discussion

We have shown that ESCMNs transplanted into the transected tibial nerve after muscle denervation generate a local neuronal circuit resulting in activity ranging from spontaneous rhythmicity to stimulus-evoked bursting. These self-assembled networks are glutamate-dependent and are modulated by GABAergic/glycinergic and cholinergic inputs. Addition of neurochemicals that lead to locomotor activity in the spinal cord, NMDA, 5-HT and DA, lengthens bursting episodes, and increases contraction forces and burst frequencies. But sustained spontaneous rhythmic output was not produced. That is, addition to ES cells of signaling factors that in the embryo lead to ventral spinal neuronal differentiation leads to a variable degree of self-assembly of microcircuits.

Embryonic stem cell-derived neurons can be readily generated *in vitro* and several protocols favor enrichment of selective neuronal subpopulations (Lee et al., 2000; Westmoreland et al., 2001; Barberi et al., 2003; Peljto and Wichterle, 2011). But despite this enrichment, a wide range of neuronal subtypes remains (Strübing et al., 1995; Lee et al., 2000; Barberi et al., 2003; Bibel et al., 2004; Nakayama et al., 2004; Park et al., 2005). The MN differentiation protocol with sonic hedgehog and retinoic acid (Wichterle et al., 2002) generates about 20 to 30% MNs as well as different interneuron types: glutamatergic (10%), GABAergic (15%), and glycinergic (6%, (Deshpande et al., 2006). Although the role of the ESCMNs in transplantation has been defined (Yohn et al., 2008), activity of the interneuron types has not.

Embryonic stem cell-derived neurons have shown to integrate within a formed network either *in vitro* in organotypic slices (Benninger et al., 2003), or *in vivo* (Lee et al., 2000; Wernig et al., 2004; Rüschemschmidt et al., 2005). Yet, little is known about the generation of neuronal networks among ES cell-derived neurons maintained *in vitro* or after transplantation.

Electrophysiological properties of ES cell-derived neurons have been investigated and shown to be similar to their embryonic equivalents functionally and anatomically (Strübing et al., 1995; Lee et al., 2000; Bibel et al., 2004; Miles et al., 2004; Nakayama et al., 2004; Toma et al., 2015). In addition, a recent study has shown the presence of intrinsically active and pacemaker neurons in *in vitro* networks formed from stem cell-derived neurons (Illes et al., 2014). Such activity may support the production of spontaneous network activity that closely resembles *in vivo* activity, as shown in studies of hippocampal networks (Ban et al., 2007; Heikkilä et al., 2009). The degree to which such activity is seen in ESCMNs and the roles – positive or negative – of such activity are not clear.

Embryonic Spontaneous Activity

Spontaneous activity is an essential component for the development of embryonic neural networks (Marder and Rehm, 2005; Blankenship and Feller, 2009) and is involved in various roles, including neurite outgrowth (Metzger et al., 1998), maturation of electrical properties (Xie and Ziskind-Conhaim, 1995), and synaptogenesis and axon pathfinding (Hanson and Landmesser, 2004; 2006; Hanson et al., 2008).

Different transmitter systems may have different effects during development. Early assembly of spinal locomotor circuits is dependent on cholinergic inputs (Myers et al., 2005). Spontaneous activity in the embryonic chick was postulated to originate from cholinergic MNs (Ritter et al., 1999) forming synapses with R-interneurons (Renshaw-like) via axon collaterals (Wenner and O'donovan, 2001). Spontaneous motor activity in the embryonic spinal cord has also been linked to presynaptic facilitation of GABA and glutamate release by cholinergic inputs (Czarnecki et al., 2014). The roles of these different transmitter systems may differ at different times of development. In the early phase of embryonic network activity, bursting is dependent on GABAergic and cholinergic transmission, while glutamatergic effects on activity only occur at

later stages of development (Branchereau et al., 2002; Hanson and Landmesser, 2003; Myers et al., 2005; Scain et al., 2010). Thus multiple transmitter systems play roles in spontaneous activity at different times during development.

The spontaneous muscular activity we observed occurred late after transplantation, and was largely glutamate-dependent, corresponding to glutamatergic predominance in late embryonic development (Branchereau et al., 2002; Hanson and Landmesser, 2003; Myers et al., 2005; Scain et al., 2010). It is possible that earlier on in the transplants, there was spontaneous activity that was insufficient to produce notable muscle contractions. Therefore, other transmitter systems may have been critical to set the stage for circuit formation.

At three months following transplantation, the residual activity seen after blockade of GABAergic/glycinergic, glutamatergic, and muscarinic cholinergic transmission could have resulted from spontaneous MN activity, nicotinic transmission (Czarnecki et al., 2014), electrical synapses, or other neurotransmitters such as dopamine or serotonin. It is also possible that higher drug concentrations of CNQX and APV or bicuculline and strychnine may have achieved a complete blockade. Nevertheless, the dominant neurotransmitter is glutamate, almost certainly released from excitatory interneurons.

Whether synapses are formed in these conditions, or transmitter is released into the local environment is not clear. Under our model conditions, transplanted ESCMNs mature in a foreign environment – a peripheral nerve disconnected from central nervous system inputs. The absence of astrocytes might have an impact on synapse formation and elimination (Christopherson et al., 2005; Stevens et al., 2007). Interestingly, neurite pathfinding early in spinal cord development is to a degree genetically determined and leads to proper location targeting even in the absence of the targeted cells (Vrieseling and Arber, 2006) as seen in la

afferent synapses onto MNs (Sürmeli et al., 2011). These cues may be absent in our transplantation model and consequently synaptic targeting may be somewhat “random.” The diversity of spontaneous activity patterns encountered in our transplants might be the result of such arbitrary synaptic formation or due to non-synaptic transmitter release.

Functional Considerations

Investigating spontaneous activity of ES cell-derived neurons could extend our understanding of neuronal developmental physiology and network formation (Ban et al., 2007; Heikkilä et al., 2009; Illes et al., 2014). Such knowledge could provide insight into the impacts of transplanted stem cell-derived neurons on the host network, some of which may be unwanted and of clinical significance (Weerakkody et al., 2013; Illes et al., 2014). Whether the microcircuit formation that resulted in spontaneous activity observed here plays an important role in the functional integration of the transplants, and/or whether it produces clinically undesirable effects remains to be seen.

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Ethics

The Dalhousie Animal Use Committee, in accordance with the standards of the Canadian Council of Animal Care, approved all procedures.

Author Contributions

PM, VRF, and RMB contributed to the conception and design of the study, PM acquired and analyzed the data, and PM, VRF, and RMB wrote the manuscript.

Conflicts Of Interest

The authors declare no competing financial interests.

References

- Arnold FJL, Hofmann F, Bengtson CP, Wittmann M, Vanhoutte P, Bading H.** Microelectrode array recordings of cultured hippocampal networks reveal a simple model for transcription and protein synthesis-dependent plasticity. *J Physiol (Lond)* 564: 3–19, 2005.
- Ban J, Bonifazi P, Pinato G, Broccard FD, Studer L, Torre V, Ruaro ME.** Embryonic stem cell-derived neurons form functional networks in vitro. *Stem Cells* 25: 738–749, 2007.
- Barberi T, Klivenyi P, Calingasan NY, Lee H, Kawamata H, Loonam K, Perrier AL, Bruses J, Rubio ME, Topf N, Tabar V, Harrison NL, Beal MF, Moore MAS, Studer L.** Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat Biotechnol* 21: 1200–1207, 2003.
- Benninger F, Beck H, Wernig M, Tucker KL, Brüstle O, Scheffler B.** Functional integration of embryonic stem cell-derived neurons in hippocampal slice cultures. *Journal of Neuroscience* 23: 7075–7083, 2003.
- Bibel M, Richter J, Schrenk K, Tucker KL, Staiger V, Korte M, Goetz M, Barde Y-A.** Differentiation of mouse embryonic stem cells into a defined neuronal lineage. *Nat Neurosci* 7: 1003–1009, 2004.
- Blankenship AG, Feller MB.** Mechanisms underlying spontaneous patterned activity in developing neural circuits. *Nat Rev Neurosci* 11: 18–29, 2009.
- Branchereau P, Chapron J, Meyrand P.** Descending 5-hydroxytryptamine raphe inputs repress the expression of serotonergic neurons and slow the maturation of inhibitory systems in mouse embryonic spinal cord. *Journal of Neuroscience* 22: 2598–2606, 2002.
- Christopherson KS, Ullian EM, Stokes CCA, Mallowney CE, Hell JW, Agah A, Lawler J, Moshier DF, Bornstein P, Barres BA.** Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120: 421–433, 2005.
- Czarnecki A, Le Corrond H, Rigato C, Le Bras B, Couraud F, Scain AL, Allain AE, Mouffle C, Bullier E, Mangin JM, Branchereau P, Legendre P.** Acetylcholine Controls GABA-, Glutamate-, and Glycine-Dependent Giant Depolarizing Potentials that Govern Spontaneous Motoneuron Activity at the Onset of Synaptogenesis in the Mouse Embryonic Spinal Cord. *J Neurosci* 34: 6389–6404, 2014.
- Deshpande DM, Kim Y-S, Martinez T, Carmen J, Dike S, Shats I, Rubin LL, Drummond J, Krishnan C, Hoke A, Maragakis N, Shefner J, Rothstein JD, Kerr DA.** Recovery from paralysis in adult rats using embryonic stem cells. *Ann Neurol* 60: 32–44, 2006.
- Gomez TM, Spitzer NC.** In vivo regulation of axon extension and pathfinding by growth-cone calcium transients. *Nature* 397: 350–355, 1999.
- Gonzalez-Islas C, Garcia-Bereguian MA, O'Flaherty B, Wenner P.** Tonic nicotinic transmission enhances spinal GABAergic presynaptic release and the frequency of spontaneous network activity. *Dev Neurobiol* (June 23, 2015). doi: 10.1002/dneu.22315.

Hammond MW, Xydas D, Downes JH, Bucci G, Becerra V, Warwick K, Constanti A, Nasuto SJ, Whalley BJ. Endogenous cholinergic tone modulates spontaneous network level neuronal activity in primary cortical cultures grown on multi-electrode arrays. *BMC Neurosci* 14: 38, 2013.

Hanson MG, Landmesser LT. Characterization of the circuits that generate spontaneous episodes of activity in the early embryonic mouse spinal cord. *Journal of Neuroscience* 23: 587–600, 2003.

Hanson MG, Landmesser LT. Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules. *Neuron* 43: 687–701, 2004.

Hanson MG, Landmesser LT. Increasing the frequency of spontaneous rhythmic activity disrupts pool-specific axon fasciculation and pathfinding of embryonic spinal motoneurons. *J Neurosci* 26: 12769–12780, 2006.

Hanson MG, Milner LD, Landmesser LT. Spontaneous rhythmic activity in early chick spinal cord influences distinct motor axon pathfinding decisions. *Brain Research Reviews* 57: 77–85, 2008.

Heikkilä TJ, Ylä-Outinen L, Tanskanen JMA, Lappalainen RS, Skottman H, Suuronen R, Mikkonen JE, Hyttinen JAK, Narkilahti S. Human embryonic stem cell-derived neuronal cells form spontaneously active neuronal networks in vitro. *Exp Neurol* 218: 109–116, 2009.

Illes S, Jakab M, Beyer F, Gelfert R, Couillard-Despres S, Schnitzler A, Ritter M, Aigner L. Intrinsically Active and Pacemaker Neurons in Pluripotent Stem Cell-Derived Neuronal Populations. *Stem Cell Reports* 2: 323–336, 2014.

Jiang Z, Rempel J, Li J, Sawchuk MA, Carlin KP, Brownstone RM. Development of L-type calcium channels and a nifedipine-sensitive motor activity in the postnatal mouse spinal cord. *Eur J Neurosci* 11: 3481–3487, 1999.

Kirkby LA, Sack GS, Firl A, Feller MB. A role for correlated spontaneous activity in the assembly of neural circuits. *Neuron* 80: 1129–1144, 2013.

Lee S-H, Lumelsky N, Studer L, Auerbach JM, McKay RD. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* 18: 675–679, 2000.

Marder E, Rehm KJ. Development of central pattern generating circuits. *Curr Opin Neurobiol* 15: 86–93, 2005.

Metzger F, Wiese S, Sendtner M. Effect of glutamate on dendritic growth in embryonic rat motoneurons. *J Neurosci* 18: 1735–1742, 1998.

Miles GB, Yohn DC, Wichterle H, Jessell TM, Rafuse VF, Brownstone RM. Functional properties of motoneurons derived from mouse embryonic stem cells. *Journal of Neuroscience* 24: 7848–7858, 2004.

Myers CP, Lewcock JW, Hanson MG, Gosgnach S, Aimone JB, Gage FH, Lee K-F, Landmesser LT, Pfaff SL. Cholinergic input is required during embryonic development to mediate proper assembly of spinal locomotor circuits. *Neuron* 46: 37–49, 2005.

Nakayama T, Momoki-Soga T, Yamaguchi K, Inoue N. Efficient production of neural stem cells and neurons from embryonic stem cells. *Neuroreport* 15: 487–491, 2004.

Park C-H, Minn Y-K, Lee J-Y, Choi DH, Chang M-Y, Shim J-W, Ko J-Y, Koh H-C, Kang MJ, Kang JS, Rhie D-J, Lee Y-S, Son H, Moon SY, Kim K-S, Lee S-H. In vitro and in vivo analyses of human embryonic stem cell-derived dopamine neurons. *J Neurochem* 92: 1265–1276, 2005.

Peljto M, Wichterle H. Programming embryonic stem cells to neuronal subtypes. *Curr Opin Neurobiol* 21: 43–51, 2011.

Ritter A, Wenner P, Ho S, Whelan PJ, O'donovan MJ. Activity patterns and synaptic organization of ventrally located interneurons in the embryonic chick spinal cord. *J Neurosci* 19: 3457–3471, 1999.

Rosato-Siri MD, Zoccolan D, Furlan F, Ballerini L. Interneurone bursts are spontaneously associated with muscle contractions only during early phases of mouse spinal network development: a study in organotypic cultures. *Eur J Neurosci* 20: 2697–2710, 2004.

Rüschenschmidt C, Koch PG, Brüstle O, Beck H. Functional properties of ES cell-derived neurons engrafted into the hippocampus of adult normal and chronically epileptic rats. *Epilepsia* 46 Suppl 5: 174–183, 2005.

Scain A-L, Le Corrond H, Allain A-E, Muller E, Rigo J-M, Meyrand P, Branchereau P, Legendre P. Glycine release from radial cells modulates the spontaneous activity and its propagation during early spinal cord development. *Journal of Neuroscience* 30: 390–403, 2010.

Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SWM, Barres BA. The classical complement cascade mediates CNS synapse elimination. *Cell* 131: 1164–1178, 2007.

Streit J, Tschertner A, Heuschkel MO, Renaud P. The generation of rhythmic activity in dissociated cultures of rat spinal cord. *Eur J Neurosci* 14: 191–202, 2001.

Strübing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM. Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. *Mech Dev* 53: 275–287, 1995.

Sürmeli G, Akay T, Ippolito GC, Tucker PW, Jessell TM. Patterns of spinal sensory-motor connectivity prescribed by a dorsoventral positional template. *Cell* 147: 653–665, 2011.

Toma JS, Shettar BC, Chipman PH, Pinto DM, Borowska JP, Ichida JK, Fawcett JP, Zhang Y, Eggan K, Rafuse VF. Motoneurons derived from induced pluripotent stem cells develop mature phenotypes typical of endogenous spinal motoneurons. *Journal of Neuroscience* 35: 1291–1306, 2015.

Van Pelt J, Corner MA, Wolters PS, Rutten WLC, Ramakers GJA. Longterm stability and developmental changes in spontaneous network burst firing patterns in dissociated rat cerebral cortex cell cultures on multielectrode arrays. *Neurosci Lett* 361: 86–89, 2004.

- Vrieseling E, Arber S.** Target-induced transcriptional control of dendritic patterning and connectivity in motor neurons by the ETS gene *Pea3*. *Cell* 127: 1439–1452, 2006.
- Weerakkody TN, Patel TP, Yue C, Takano H, Anderson HC, Meaney DF, Coulter DA, Wolfe JH.** Engraftment of nonintegrating neural stem cells differentially perturbs cortical activity in a dose-dependent manner. *Mol Ther* 21: 2258–2267, 2013.
- Wenner P, O'donovan MJ.** Mechanisms that initiate spontaneous network activity in the developing chick spinal cord. *J Neurophysiol* 86: 1481–1498, 2001.
- Wernig M, Benninger F, Schmandt T, Rade M, Tucker KL, Büssow H, Beck H, Brüstle O.** Functional integration of embryonic stem cell-derived neurons in vivo. *Journal of Neuroscience* 24: 5258–5268, 2004.
- Westmoreland JJ, Hancock CR, Condie BG.** Neuronal development of embryonic stem cells: a model of GABAergic neuron differentiation. *Biochemical and Biophysical Research Communications* 284: 674–680, 2001.
- Wichterle H, Lieberam I, Porter JA, Jessell TM.** Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110: 385–397, 2002.
- Xie H, Ziskind-Conhaim L.** Blocking Ca(2+)-dependent synaptic release delays motoneuron differentiation in the rat spinal cord. *J Neurosci* 15: 5900–5911, 1995.
- Yohn DC, Miles GB, Rafuse VF, Brownstone RM.** Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy. *Journal of Neuroscience* 28: 12409–12418, 2008.

Figure Legends

Movie 1 – Spontaneous Muscle Contractions Induced From Transplanted ESCMNs In The Tibial Nerve

Ex vivo transplanted tibial nerve under surgical microscope. Enlargement at the end of nerve represents the transplantation site. Top muscle is the medial gastrocnemius spontaneously contracting. Femur is anchored on the left with pins. Suction electrode on top of muscle for EMG recording. Stimulating electrode at the bottom is not contacting the nerve and not stimulating.

See appendix two

Figure 1 – Transplanted ESCMNs Generate A Neuronal Circuit Resulting In Rhythmic Muscle Contractions

(A) Bursting activity evoked after three 5 Hz stimuli over 500 ms. Evoked activity was blocked after the addition of CNQX and APV. Arrows represent electrical stimuli. (B) Spontaneous muscle contractions at baseline, after CNQX and APV infusion, and after washout. Spontaneous contractions significantly reduced after CNQX and APV infusion with residual low amplitude contractions shown in the insert. Grey bars indicate region of insets. (C) Quantification of force and instantaneous contraction frequency before and after CNQX and APV. * false discovery rate significant, $Q < 1\%$. (D) Autocorrelation of baseline, CNQX and APV, and washout conditions. (E) Histogram of force frequencies before and after addition of CNQX and APV. Only few amplitude events are present before blockade whereas no large amplitude events are seen after blockade. (F) Addition of GABA and glycine blockers, bicuculline and strychnine, resulted in an increase in force (H) early (G) but not late after infusion of GABA and glycine blockers. Contraction instantaneous frequency did not change (H). Grey bars (F) indicate regions depicted in (G). **** $p < 0.0001$ by unpaired t-test, $n = 1533$ vs 117 events. (I) Autocorrelation of baseline (solid black), early GABA / glycine blockade (dotted grey) and late GABA / glycine blockade (solid grey). Rhythmicity can be seen after prolonged GABA / glycine blockade.

Figure 2 – Examples Of Variety Of Responses To Atropine Demonstrated By Transplanted ESCMNs

(A) Evoked bursting activity at baseline and after atropine infusion. Duration of bursting was prolonged after the addition of atropine. Arrows represent electrical stimuli (25 pulses at 50 Hz). (B) Quantification of burst duration. * $p = 0.03$, Mann Whitney test, $n = 1$, 4 repeats. (C) Spontaneous activity at baseline with few large amplitude events (force ≥ 6 mN). The addition of atropine increased the frequency of large amplitude events. (D) Low amplitude spontaneous events (force < 6 mN) were present at baseline and reduced in frequency after atropine. (E) Quantification of inter-event intervals between all events, small events only and large events only. There is a four fold decrease in the inter-event interval of large events. **** $p < 0.0001$ (F) Frequency distribution of instantaneous event frequency at baseline and after atropine. Atropine narrows the distribution towards 0.5 Hz. (G) Quantification of force shows no change in amplitude. (H) Frequency distribution of forces before and after atropine. Atropine decreases the occurrence of low amplitude events and increases large amplitude events. Inset represents enlarged region containing large amplitude events. (I) Neurotransmission blockade with CNQX, APV, bicuculline and strychnine shows residual low amplitude activity. The addition of atropine resulted in a decrease of spontaneous activity. **** $p < 0.0001$ (J) Quantification of force shows no change in amplitude, but (I) atropine significantly decreases the event instantaneous frequency. **** $p < 0.0001$

Figure 3 – Transplants Demonstrating Bursting Activity Do Not Become Spontaneously Rhythmically Active After Adding Excitatory Neurotransmitters

(A) The addition of NMDA, 5-HT and DA resulted in an increase activity demonstrated by a prolongation of burst duration in evoked activity. (B) Quantification of episode duration. ** $p = 0.004$, Mann-Whitney test, 8 vs. 4 repeats. (C) Autocorrelation before and after NMDA, 5-HT

and DA shows the onset of rhythmic activity. (D) In transplants with spontaneous activity, the addition of NMDA, 5-HT and DA increase the force of events, the instantaneous event frequency, and decreased the inter-event duration. Two examples of force generated at baseline are shown in baseline (solid and dotted line) to represent the presence of single events and short bursting episodes. (E) Quantification of force amplitude, (F) instantaneous frequency, and (G) inter-event intervals. *** $p = 0.0004$ in (D) and $p = 0.0002$ in (E), unpaired t-test with Welch's correction, 20 vs 399 events, ** $p = 0.01$ in (G)

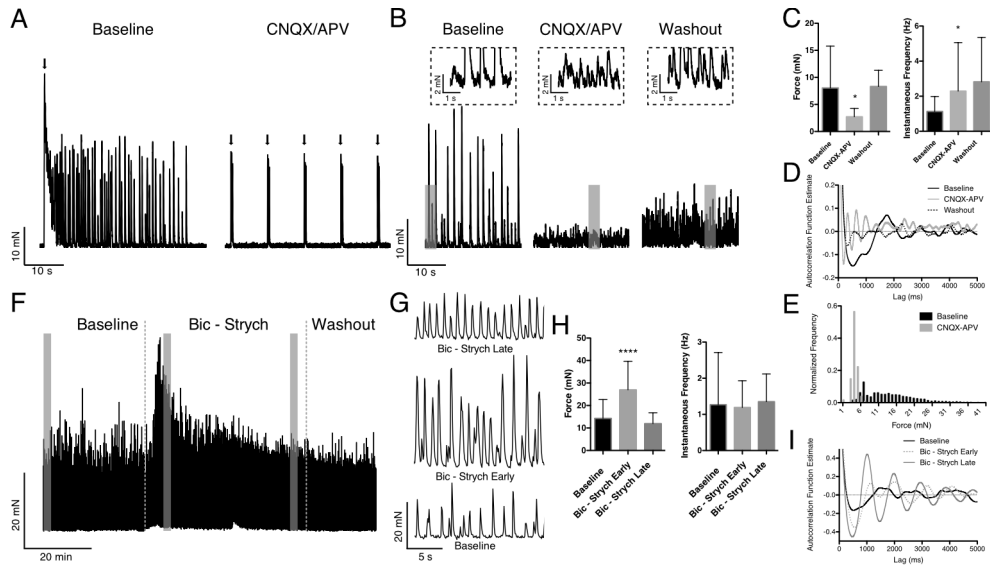


Figure 1 – Transplanted ESCMNs generate a neuronal circuit resulting in rhythmic muscle contractions

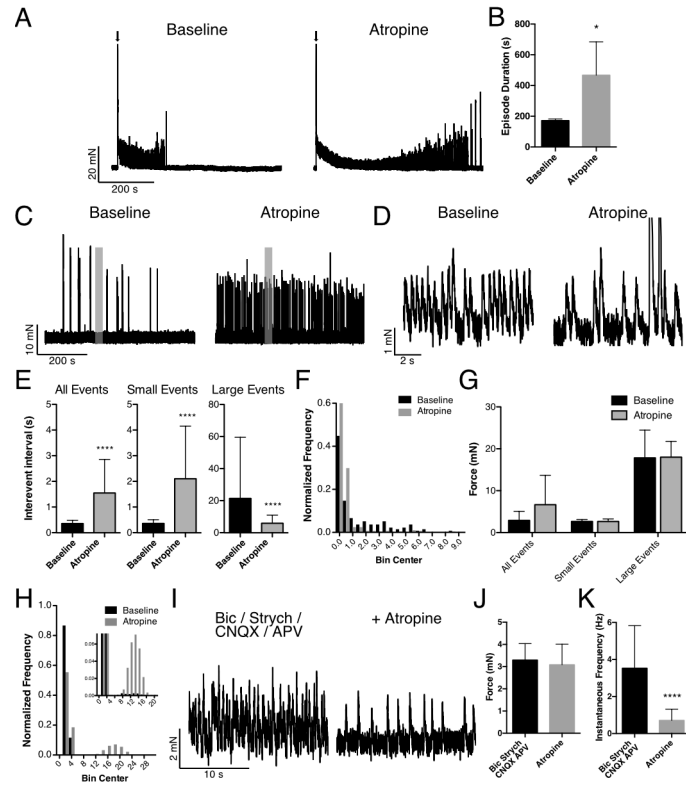


Figure 2 – Examples of variety of responses to atropine demonstrated by transplanted ESMCNs

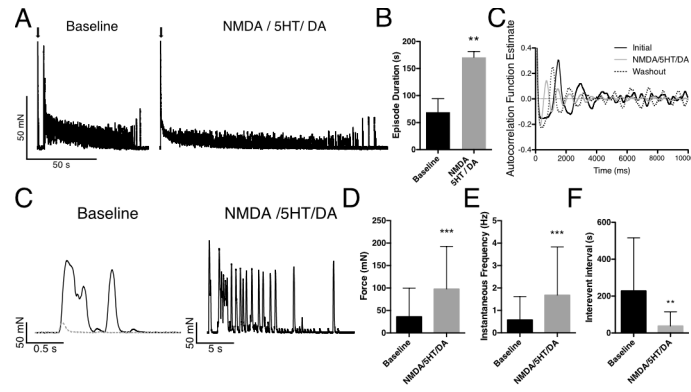


Figure 3 – Transplants demonstrating bursting activity do not become spontaneously rhythmically active after adding excitatory neurotransmitters

Chapter Five: Discussion

Summary

Chapter Two: Preventing Tumorigenesis Improves Innervation By Transplanted Embryonic Stem Cell Derived Motoneurons

In a clinically relevant model of delayed surgical intervention for denervation pathologies, we found that muscle fibre innervation by transplanted ESCMNs decreased progressively over four weeks and failed by eight weeks. Concurrently, we found that about half of transplants developed teratocarcinomas arising from residual pluripotent cells within the graft. We addressed tumorigenesis by pre-treating our cultures with the antimitotic agent mitomycin C. We found that mitomycin C treatment prevented tumor formation and simultaneously improved muscle innervation from transplanted ESCMNs.

Chapter Three: Direct Optical Activation Of Skeletal Muscle Fibres Efficiently Controls Muscle Contraction And Attenuates Denervation Atrophy Due To Injury

Return of muscle innervation post denervation being limited to a finite time window, we focused on activation of muscle contraction via direct depolarization of muscle fibres. Mutant Sim1-cre: Ai32 mice expressed the ChR2-H134R homogeneously along the sarcoplasmic membranes. Upon light stimulation with an "off the shelf" 470 nm LED, we replicated normal contractile forces and were able to modulate force production with light intensity, duration, and pulse frequency.

Chapter Four: Spontaneous Circuit Activity Of Transplanted Embryonic Stem Cell-Derived Motoneurons

Transplantation of ESCMNs in the transected tibial nerve generated a local neuronal circuit resulting in a variable range of activity from spontaneous rhythmicity to bursting of contractions evoked by stimulation. This self-generated network required glutamatergic transmission and could be modulated by GABAergic and cholinergic transmission. Increasing the network excitability with NMDA, 5-HT and DA lengthens bursting episode, contraction force and frequency but was not enough to transform the network into a spontaneously bursting circuit. This unexpected self-organized neuronal circuit formed by transplanted ESCMNs raises concerns on outcomes after transplantation of embryonic neurons in a non-native environment where guidance cues required for proper elaboration of a neuronal synaptic network are likely unregulated.

Motor Recovery

The number of MNs needed to reinnervate a muscle is fewer than the native number, as MNs are able to quintuplet their innervation ratio (Rafuse et al., 1992). In the absence of a sufficient number of MNs, however, compensation saturates and muscle fibres left rapidly degenerate (Bradley et al., 1983; Thomas et al., 1997). Extensive MN death thus leads to poor functional outcomes irrespective of the etiology (Thomas et al., 1997; Jackson and Bryan, 1998; Martin et al., 1999; Thomas et al., 2000; Reier, 2004; Collazos-Castro et al., 2005; Ramer et al., 2005). Return of innervation can occur within a finite time window, outside of which functional recovery is poor (Sunderland, 1978; Irintchev et al., 1990; Fu and Gordon, 1995a; Finkelstein et al., 1996; Kobayashi et al., 1997; Ruijs et al., 2005; Isaacs, 2010).

To this end, transplantation of MNs into either the spinal cord grey matter or the peripheral nervous system has been investigated and has demonstrated successful innervation of muscles (Sieradzan and Vrbová, 1989; Horvat et al., 1991; Sieradzan and Vrbová, 1991; Deshpande et

al., 2006; Nógrádi and Szabó, 2008). Transplantation into the peripheral nervous system has been used to eliminate inhibition of axonal growth by central myelin, to shorten the distance and thus the time needed for axonal growth and enhance engraftment by transplanting in an atraumatic environment.

Force generation in our transplants performed immediately (Yohn et al., 2008) or after prolonged denervation recovered to 55 to 65% of naive MG forces. Partial force recovery after transplantation is commonly reported among laboratories working with this transplantation model. Practically, a 50% force recovery renders a reinnervated muscle functional as it can generate enough power to resist at least gravity. In humans, results of peripheral nerve graft and transfer for high grade injuries are commonly limited to return of anti-gravity power in successful candidates (Yang et al., 2012). Our peripheral nerve transplantation model seems advantageous. However, force recovery might be drastically different among species. In fact, in the rat, force recovery after transplantation of MNs in the tibial nerve is only about 5% (0.24 ± 0.09 N vs. 4.6 ± 0.3 N in transplanted and naive rat MG respectively; Grumbles et al., 2005).

We estimated that after transplantation, about 15 MNs innervated the MG. The mouse MG normally receives ~ 50 MUs (Mohajeri et al., 1998; Yohn et al., 2008). Transplanted ESCMNs providing innervation accounted for 30% of the normal number of MNs. With such a high number, it would have been expected that more than 100% of the force would have been restored based on MN sprouting capacity. On the contrary, transplanted ESCMNs generated half of the expected force. There are several possible explanations why this was not the case: (1) muscle wasting from disuse atrophy; (2) change in muscle fibre type phenotype; or (3) impaired sprouting capacity of ESCMNs.

Disuse atrophy, demonstrated in patients after spinal cord injury, results in a 50% decrease in force generation in innervated muscles distal to the injury (Thomas et al., 1997). Given that transplanted ESCMNs remained disconnected from the central nervous system, we assume that the MG remained mostly inactive and was thus prone to atrophy from disuse. Interestingly, the averaged MU force after transplantation was similar to normal controls. This should not be the case in disuse atrophy, in which MU force is halved the normal value. In our model, on the other hand, muscle fibres being denervated, MNs would have been strongly promoted to sprout. Muscle units should theoretically have enlarged. Unfortunately, muscle unit size and muscle fibre cross-sectional areas were not investigated in our transplants. On the other hand, MG wet weights from transplants were halfway between the weights of normal and denervated MG (17 mg vs. 34 mg vs. 53 mg, denervated, transplanted and normal respectively), suggesting a blunted loss of mass likely from neurotrophic control by transplanted MNs assuming a lack muscle contraction. Together, these findings would be consistent with disuse atrophy.

We have previously demonstrated a change in fibre type from fast to slow when ESCMNs innervate the MG (Yohn et al., 2008). While slow fibres have lower force output, generally the most prominent change from fast to slow conversion is in force kinetics (Tötösy de Zepetnek et al., 1992). In fact, denervation induces a spectrum of changes in muscle fibre physiology including disruption of calcium handling (Berchtold et al., 2000) and blurring of muscle fibres contractile phenotype (Celichowski et al., 2006; Patterson et al., 2006; Mrówczyński et al., 2011). In the absence of contractile activity, these changes may not have fully recovered after reinnervation and might have contributed to poor force generation.

Embryonic stem cell-derived motoneurons may have a reduced capacity to sprout. We have no evidence for a cell autonomous sprouting impairment. However, activity driven by spinal or supraspinal inputs may be an essential drive underlying sprouting (Rutishauser and

Landmesser, 1991; Hanson and Landmesser, 2004; Burns et al., 2007). Absence of these inputs may reduce ESCMNs capacity to sprout.

Alternatively, muscle end plates might not be able to receive innervation preventing sprouting axons to establish a functional NMJ (Ma et al., 2011). Results from transplantation of ventral cord dissociated neurons hints towards a failure of forming functional NMJ despite sprouting capacity (Grumbles et al., 2005; 2012). In that model, transplanted rats had 12 MUs compared to 82 MUs in normal controls irrespective of the overwhelming number of neurons transplanted (10^5 compared to 10^6). Surviving MNs should maximally sprout to expand their innervation ratio. In that case, only 18 MUs would be required to recover full force. With 12 MUs, two third of normal force should have be recovered, transplanted rats generated only 5% of normal force. Even while accounting for disuse atrophy causing a 50% loss of force, recovery would only have been 10% of total force (0.23 N vs. 4.6 N, transplant vs. normal). Yet, 50% of MG fibres were received innervation after transplantation. Together, these findings are most consistent with a problem along functional synapses formation. This would be consistent with the immature morphology seen in 80% of synapses formed by transplanted embryonic MNs (Grumbles et al., 2002; 2012).

Timing Of Transplantation

Others and us have found that a delay of one week before transplantation resulted in optimal recovery (Grumbles et al., 2002; Lin et al., 2013). At one week, the inflammatory environment resulting from the transection has likely transformed to a restorative milieu rich in neurotrophic factors and axonal growth promoting substrate produced by activated SCs (Richardson and Issa, 1984; Perry and Brown, 1992; Rassendren et al., 1992; deLapeyrière and Henderson, 1997; Grumbles et al., 2002). Given that the rate of cellular death in the grafts is highest at the

time of transplantation (Sortwell et al., 2000), these survival signals may lead to improvement in the outcomes.

Motor Regeneration After Prolonged Denervation

The failure of recovery after prolonged denervation is linked to a failure of axons to reach the target to establish a functional synapse (Fu and Gordon, 1995b; 1995a). The denervated muscle and the nerve leading to it are progressively down-regulating production of growth supportive factors (Gordon and Fu, 1997; Höke et al., 2002). The distal milieu becomes non-permissive for growth (Sulaiman and Gordon, 2000) and eventually promotes axonal retraction (Luo and O'Leary, 2005; Gallo, 2006). Returning axons maintain an immature morphology at the endplate with minimal branching and fail to form synaptophysin expressing terminals (Ma et al., 2011). Based on these findings, a shorter window of reinnervation of five weeks has been demonstrated in mice beyond which axons fail to establish a functional NMJ (Ma et al., 2011; Sakuma et al., 2015). This finding focusing reinnervation failure at the NMJ would be more in keeping with studies showing motor axons maintaining an intrinsic capacity to grow despite prolonged axotomy (Fu and Gordon, 1995b; Terenghi, 1999; Saito and Dahlin, 2008).

The repeated crush injury model in the mouse indicates that return of innervation must occur before 31 days after which recovery drops by 50% and there is no recovery if innervation is delayed to 38 days (Ma et al., 2011; Sakuma et al., 2015). Full recovery should be expected if innervation returns by three weeks post injury and would fail if returning after five weeks. Similar results were found after transection and resuturing of the tibial nerve showing return of innervation to the triceps surae by 20 days. A failure of motor recovery in distal foot intrinsic muscles was also demonstrated (Sakuma et al., 2015). Assuming a growth rate of 1 mm/day, axons would need an extra two weeks to reach the intrinsic foot musculature, and by that time, the muscles would have already been denervated for 35 days.

In summary, prolonged denervation is species specific. In the mouse, return of innervation must occur before 35 days (five weeks). Further delays results in quasi-null recovery. A transection injury immediately repaired cause a denervation of four-week duration. A repair delayed by two week or longer would push the return of innervation beyond this critical window. Although previous studies have demonstrated muscle contractions despite prolonged denervation periods, the force outputs reported have remained poor (Carraro et al., 2015).

Denervation Atrophy

Muscle atrophy is a subtype of muscle mass loss (Dutt et al., 2015). Loss of muscle mass can originate from three major conditions: (1) chronic disease named cachexia; (2) disuse conditions, or atrophy from denervation, immobilization and microgravity; and (3) aging represented by sarcopenia (Dupont-Versteegden, 2005; Glass, 2005; Zhang et al., 2007).

Essentially, the regulation of muscle mass reflects protein turnover. Muscle atrophy occurs when protein degradation rates exceed protein synthesis (Schiaffino et al., 2013). Muscle atrophy involves shrinkage of myofibres due to a net loss of proteins, organelles and cytoplasm. Contrarily to other atrophic conditions, denervation atrophy is associated with a cell-wide increase in protein synthesis with an even more important increase in protein degradation (Argadine et al., 2009; Quy et al., 2013). Muscle fibre subtypes do not respond equally to atrophic conditions and individual muscles show different response to denervation (Aravamudan et al., 2006).

Nervous System Control Of Muscle Mass

The nervous system exerts control over skeletal muscles by two mechanisms: neuromotor control, inducing depolarization-contraction coupling, and neurotrophic control, which is independent of the electrical activity of MNs, but depends on the release of soluble factors from

the nerve terminals at the NMJ. The importance of neural influences on skeletal muscle is evident from the rapid and severe muscular atrophy that occurs whenever there is loss of neural continuity (Zeman et al., 2009); the ensuing atrophy is considerably more rapid than from other etiologies such as immobilization, cachexia, malnutrition, severe burns, aging, dystrophies, and myasthenia gravis (Schiaffino et al., 2013). Application of electrical stimulation to nerves to elicit muscle contractions can prevent or largely reverse muscle wasting due to paralysis indicating the critical role of muscle contraction in suppressing the signaling responsible for muscle atrophy (Dudley-Javoroski and Shields, 2008).

There is also a vital neurotrophic role provided by an intact MN at the NMJ, as demonstrated by slower muscle atrophy after spinal isolation compared to a rapid atrophy following nerve transection (Hyatt et al., 2003). Maintaining neurotrophic support of denervated muscle by temporary innervation from a grafted sensory nerve also improves functional recovery, pointing towards the influence of neurotrophic support on prolonging the capacity of NMJs to receive innervation (Bain et al., 2001; Zhao et al., 2004).

Nerve transection increases sarcolemma permeability, reducing membrane potential, and increasing excitability of muscle fibres. Most of these changes have recently been proposed to result from de novo synthesis and insertion of connexins 39, 43 and 45, as well as pannexin 1 into the sarcolemma, and is responsible to mediate atrophy of fast skeletal muscle (Cea et al., 2013). Connexin expression is normally down regulated before the end of myogenesis, such that adult myocytes are devoid of gap junctions (Armstrong et al., 1983; Constantin and Cronier, 2000). However, regenerating muscle fibres re-express connexins as early as seven days post-denervation and as late as eight weeks post SCI (Araya et al., 2004; Maltzahn et al., 2004; Belluardo et al., 2005; Cea et al., 2013). Knocking out connexins 43 and 45 prevents muscle atrophy post denervation by 70% (Cea et al., 2013).

Muscle Atrophy After Spinal Cord Injury

In rodents and humans, SCIs result in rapid and extensive muscle atrophy (Qin et al., 2010) causing 40-60% muscle loss in rodents (Ung et al., 2010; Wu et al., 2012) and 27 to 56% in humans (Castro et al., 1999). These changes are associated with marked reductions in contractile force and fatigue resistance, loss of slow- and fast-twitch oxidative fibres, and diminished levels of oxidative phosphorylation enzymes (Qin et al., 2010). The severity of atrophy following SCI goes beyond disuse and might be the result of changes in sarcolemma properties from an elevated expression of connexins. Interestingly, FES does not prevent the loss of membrane potential seen in muscle fibres after SCI (Squecco et al., 2009).

Changes at the NMJ are very heterogeneous: from massive sprouting to loss of acetylcholine receptor (AChR) clusters, to an absence of changes (Burns et al., 2007). Pruning of MN arborization and deterioration of motor endplates lead to impaired NMJ transmission (Ollivier-Lanvin et al., 2009). This reflects the limitation of neurotrophic control provided by MNs in preventing the biochemical changes in muscle fibres post SCI. In a way, changes seen in muscles after SCI can be expected in our transplantation model since transplanted ESCMNs are disconnected from the CNS.

Transplantation Of Motoneurons

So far, the only way to replace lost MNs is via transplantation of exogenously generated MNs. We have discussed transplantation of MNs in the peripheral nervous system already, but what happens to MNs after transplantation in the central nervous system? After transplantation of MNs into the spinal cord, it would be fundamental to demonstrate that MNs can survive in the new host post transplantation, can integrate in the circuitry of the recipient and can innervate target muscles.

Survival Of Transplanted Motoneurons

Transplantation of embryonic spinal cord enriched MNs have been shown to be possible (Sieradzan and Vrbová, 1991) but within very limited conditions: transplanted MNs must be of embryonic age, a muscle target must be available, and a matching host environment must have free space for engraftment of the transplanted MNs.

Embryonic MNs can only be harvested and transplanted within a small time window before axonal elongation and growth to the periphery (Altman and Bayer, 1984). Subsequently, embryonic MNs undergo programmed cell death, which coincide with innervation of muscle fibres (Hamburger and Oppenheim, 1990). Motoneuron survival is dependent on receiving trophic support from muscle (Hollyday and Hamburger, 1976) and this dependency extends up to early postnatal days (Lowrie and Vrbová, 1992). This biological dependency onto muscle fibre innervation is problematic for MN survival: transplanted MN would too far from their target that they would undergo apoptosis before muscle fibres. Transposition of appendage skeletal muscle and nerve to a paravertebral location solved this issue and confirmed the feasibility of spinal cord transplantation (Clowry et al., 1991; Nógrádi and Vrbová, 1996). Clinical applicability in the adult spinal cord remains questionable.

A proper matching host environment must be available such as a spinal cord devoid of MNs. SCIs and NMDs can provide such an environment but are also associated with cellular and acellular growth inhibitors that are absent in the normal spinal cord (Lu et al., 2007).

Furthermore, isogenic grafts do better and can survive in the adult spinal cord whereas allogeneic grafts eventually get rejected (Theele et al., 1996), motivating the use of patient specific iPS MNs or induced MNs.

Spinal cord grafting results are not consistent between studies. Uncontrollable factors such as graft survival, glial differentiation of transplanted NPSCs, immortalization of transplanted cells, inhibitory effects of surrounds glia and myelin, and neurotrophic and vascular factors seem to be responsible for this variability (Lu et al., 2003; Lowry et al., 2008; Abematsu et al., 2010; Lu et al., 2012). Overall, the age and source of the grafted neurons and the transplantation method result in a significant effect on cell survival, differentiation and ability to extend axons (Cao et al., 2001; Pfeifer et al., 2004; Karimi-Abdolrezaee et al., 2010; Cusimano et al., 2012).

Innervation By Grafted Motoneurons

Only a fraction (~ 20%) of axons exit the spinal cord after transplantation (Nógrádi and Vrbová, 1996; Nógrádi and Szabó, 2008). It remains unknown if grafted MN axons would have successfully exited the spinal cord in the absence of endogenous axons having already created a path (Wang et al., 2011). Furthermore, only a small portion of grafted MNs eventually expressed choline-acetyl transferase suggesting an incomplete maturation post grafting (Clowry et al., 1994). Incorporation of the grafted MNs within the pre-established spinal network remained incomplete and secretion of acetylcholine following depolarization remained non-regulated (Gulino et al., 2007). The underlying mechanism responsible for the interruption of maturation post grafting remains elusive, but might be related to deficient activity from incomplete synaptic inputs.

Connections Between The Host And The Grafted Neurons

Grafted neurons extend lengthy axons along white matter tracts after spinal cord transplantation (Bregman et al., 2002) and can extent to remarkable distances (Lepore and Fischer, 2005). They can establish reciprocal synaptic connections and assist descending pathways axons to cross the injury gap either by a bridging effect or by establishing a local circuit (Coumans et al.,

2001; White et al., 2010; Lu et al., 2012). However, most often axons fail to enter the core of the graft (Nógrádi et al., 2011).

Augmentation of axonal growth signaling pathways by trophic support or genetic manipulations have improved connectivity between the grafted neurons and the host network (Deshpande et al., 2006; Lu et al., 2012). However, transplanted neurons also formed aberrant connections onto unwanted pathways and axons misdirected into foreign locations (Canty and Murphy, 2008). Modifying axonal growth properties may result in misdirecting and unwanted synaptic connections between transplanted tissue and the host or among transplanted neurons.

Motoneuron Transplantation Preclinical Studies

Transplantation of MNs in the spinal cord after neuronal depletion resulted in 15% cell survival and engraftment but required co-transplantation of GDNF secreting neural precursors and the administration of phosphodiesterase inhibitors (Deshpande et al., 2006). Motoneurons transplanted in ALS (López-González et al., 2009) and spinomuscular atrophy (Corti et al., 2010) spinal cords delayed the onset of the disease and supported MN survival. Again, the underlying mechanisms for these effects remain unknown.

Tumour Prevention

When considering translation of stem cell therapies, it is critical to be able to prevent cancer formation (Bretzner et al., 2011). Undifferentiated stem cells have a very high propensity to form tumours (Lawrenz et al., 2004) and pre-sorting of cells is likely to be insufficient with current technology (Geens et al., 2006). Of course, future technology may be able to eliminate all pluripotent cells (Hermann et al., 2011). We used a DNA cross-linking chemotherapeutic agent, mitomycin C, which is non-selective, and relies on the greater sensitivity of pluripotent and immature cells to apoptosis compared to post-mitotic cells (Smith et al., 2012). Targeted

cytotoxic approaches developed to eradicate tumorigenic cells with ceramide analogues (Bieberich et al., 2004) or cytotoxic antibodies against specific cell surface markers (Choo et al., 2008) may provide a selective eradication of teratogenic cells.

Residual Pluripotent Cells

It should come as no surprise to encounter tumours after transplantation of undifferentiated stem cells. After all, stem cells are capable of generating all germ layer lineages upon transplantation. We initially conceptualized that differentiation would remove all pluripotent cells. Yet, as demonstrated with human ES cells, even prolong differentiation protocols do not completely remove all pluripotent cells (Brederlau et al., 2006).

This brings up an important question: are the residual proliferative SSEA-1⁺ cells that we have found after our differentiation protocol the result of a differentiation failure or a normally generated population after neuronal differentiation of cells from the epiblast / inner cell mass. Could these cells be founder cells or PGCs? The debate on the subject is controversial with serious implications for clinical trials using ES cell-derived neurons (Zwaka and Thomson, 2005; Blum and Benvenisty, 2008; Blum et al., 2009; De Miguel et al., 2009) and serious implications for the use of ES cells in clinical trials.

Maintenance of ES cells *in vitro* has been linked to adaptation and an increased risk of tumorigenesis (Baker et al., 2007). Although *in vitro* adaptation was initially reported to be due to aneuploidy and other chromosomal abnormalities, *in vitro* adapted colonies might in fact be demonstrating "tumorigenic hallmarks" in the absence of "mutational transformational hits" (Blum and Benvenisty, 2009). As such, SSEA-1⁺ cells remaining post differentiation might have been relatively refractory to differentiation without necessarily harboring oncogenic mutations. In fact, in the embryo, pluripotent cells from the inner cell mass and the epiblast have a

heterogeneous expression of pluripotent markers (Buehr et al., 2003; Rossant et al., 2003) suggesting that among them, only a minority can sustain a pluripotent state *in vitro* (De Miguel et al., 2009). This would indicate that ES cells capable of self-renewal *in vitro* are in fact a highly selected cell population prone to *in vitro* adaptation.

On the other hand, the inner cell mass gives rise to an early epiblast that, very early at 6.0 to 6.5 days post fertilization, produces primordial germ cells (PGCs; Lawson and Hage, 1994; McLaren, 2003). By 7.25 days post fertilization PGCs can be identified at the base of the allantois, an extra-embryonic mesodermal structure (Ginsburg et al., 1990). It is interesting that transplantation of embryonic structures from embryos eight days post fertilization or beyond never generates teratocarcinomas but benign teratomas (Damjanov, 1993). In fact, PGCs arise secondary to the proximal epiblast microenvironment (Quinlan et al., 1995; Tam and Zhou, 1996; Extavour and Akam, 2003) and, under the right conditions, ES cells can give rise to early PGCs (Tsang et al., 2001; Geijsen et al., 2004). Mouse ES cells differentiated by EB protocol towards a hematopoietic lineage contained a residual SSEA-1⁺ cell population (< 10%) that maintained SSEA-1 expression despite treatment with RA. Contrarily, ES cells exposed to RA prior to EB protocol differentiation rapidly and nearly totally (99%) extinguished SSEA-1 expression (Geijsen et al., 2004). This suggests that EBs provide the required microenvironment to generate PGCs. Our residual SSEA-1⁺ population found after neural differentiation protocol could have in fact been PGCs. Further maturation *in vitro* is required before PGCs can be differentiated from ES cells by the mouse germ cell marker vasa homolog (Durcova-Hills et al., 2003).

Experimental Teratocarcinomas: An Epigenetic Phenomenon

Teratocarcinomas are not well understood but these tumours have played a pivotal role in the generation of ES cells (Andrews et al., 2005). Teratocarcinomas are composed of a mixture of

differentiated tissue, similar to a teratoma, and an undifferentiated component, named embryonal carcinoma (EC), resembling embryonic tissue and responsible for the malignant behavior of these tumours (Kleinsmith and Pierce, 1964). EC cells, like ES cells, have self-renewal properties, are capable to generate cells of all germ lineages and once transplanted generate a teratocarcinoma (Kleinsmith and Pierce, 1964).

Embryonal carcinoma cells isolated from spontaneous teratocarcinomas are aneuploid with specific genetic abnormalities increasing self-renewal capacities and decreased differentiation potential (Blum and Benvenisty, 2009). However, euploid ES cells from both human and mouse are also able to generate teratocarcinomas after transplantation (Fujikawa et al., 2005; Leor et al., 2007). The formation of teratocarcinomas from embryonic tissue is dependent on the transplant site microenvironment, the recipient immune system and the recipient genetic permissiveness for teratocarcinomas (Damjanov, 1993). Consequently, to reveal teratocarcinogenic properties of human ES cells, it is essential to transplant into a human-mouse hybrid where human fetal tissue have been a priori engrafted into a SCID mouse (Shih et al., 2007). This means that stem cell-derived teratocarcinomas require an isogenic microenvironment to occur. Thus, the occurrence of teratocarcinomas was an epigenetic phenomenon by the recipient microenvironment onto PGCs residing among the transplanted ESCMN population.

Future Directions

Motoneuron Transplantation

Significant loss of MNs results in debilitation. Available medical and surgical therapies for denervation pathologies are limited and functional outcomes are only modest. TPNIs, especially proximal injuries such as plexus injuries, are associated with significant deficits and limited

functional recovery (Giuffre et al., 2010). Practically, these injuries “disconnect” the MNs from their muscle and regenerating axons fail to reach the muscle in time for full reinnervation. MNDs are a progressive denervation pathology caused by MN death. Sprouting from surviving MNs compensate temporarily until enough MNs have died that compensatory mechanisms are overwhelmed. This process eventually gives rise to permanently denervated muscle fibres. In both conditions, MNDs and TPNIs, replacing loss MNs would provide the missing innervation required to restore function. In this context, cell-based therapy employed to either replace lost MNs or support muscle fibres until return of innervation is an appealing venue for restoration of function.

To address this, we had previously transplanted ESCMNs in the mouse transected tibial nerve (Yohn et al., 2008). In that model, transplantation was done at the time of the denervation injury caused by a nerve transection. Here, we have shown that delayed transplantation of ESCMNs is possible, but is dependent on the duration of denervation and progressively decline as the denervation period lengthens.

In humans, transplantation of MNs to provide new innervation would need to be performed within a 12 months time window; time frame that should include the time required for axon to reach the muscle and form functional synapses. Expert recommendations suggest to delaying surgical procedures in humans by four to six weeks after injury to allow for spontaneous regeneration (Spinner and Kline, 2000). A six-week delay before transplantation would be unlikely to negatively impact innervation outcomes in humans. The equivalent period in the mouse model remains unknown. However, considering that the mouse do not reinnervate after eight weeks of denervation, we could postulate that eight weeks in the mouse is equivalent to 12 months in humans. Based on this premise, a six-week delay in humans would be similar to a one-week delay in mice.

Clinical translation of this transplantation model would require a few technical modifications. First, the transplantation model to be used in humans should be constructed on practiced peripheral nerve surgical approaches. Consequently, cells should not be grafted inside the nerve, but outside in an end-to-side graft procedure (Zhang and Fischer, 2002). Cells would need to reside in a substrate or a matrix that could be grafted onto the nerve side (Wong et al., 2014). In this procedure, intact endogenous axons would be spared, while grafted neurons would gain access to the nerve to grow axons.

Second, because neurons in our model are transplanted into the peripheral nervous system, isolated from the central nervous system, and thus cannot provide voluntary control over muscle contraction. In order to be functional, transplants could be fitted with a neuroprosthesis, technology currently existing for SCI and stroke patients (Gan et al., 2007). ESCMNs could be considered as “placeholders”, providing early innervation and preserving NMJs until endogenous axons return months or years later. Then, transplanted ESCMNs could be ablated permitting endogenous MNs to reinnervate the muscle.

Channelrhodopsin

The ChR2-H134R used in our Sim1-cre mouse model has allowed us to reproduce normal force with functionally minimal limitations in generating tetanic forces at high frequency stimulation. However, the H134R variant is temporally less precise than wild-type ChR2, demonstrating slower kinetics mostly a longer closing time at 18 ms (Lin et al., 2009) limiting reliability of repetitive stimulation to 50 Hz. This effect is more pronounced under overexpression and in neurons lead to action potential doublets from single pulse stimulation (Nagel et al., 2005; van Bremen et al., 2015).

One interesting future modification of the Sim1-cre: Ai32 would be to use a ChR variant with faster kinetics. Many variants of ChR are now available with different characteristics, the majority demonstrating a reduction of desensitization (Lin et al., 2009; Wen et al., 2010; Kleinlogel et al., 2011). One subtype of variants of interest is the engineered chimera of ChR1 and ChR2 named ChD, ChEF and ChIEF (Lin et al., 2009) that could improve temporal precision. All demonstrate an increased steady-state phase response with minimal of the peak photocurrent giving consistent response under continuous light exposure and repetitive light pulses at high frequencies (Lin et al., 2009). Furthermore, the kinetics of these chimeric channels are faster with a closing time of 10 ms or less, expression is enhanced in mammalian cells, and trafficking to the cell membrane is more efficient (Lin et al., 2009; Wang et al., 2009; Kleinlogel et al., 2011). Our Sim1-cre ChR2 could be modified to incorporate the ChIEF or ChD variants to improve responses at high frequency stimulation.

Finally, targeting the ChR to the NMJ might also be beneficial. As muscle fibre only need to be depolarized by 10 to 15 mV to activate the sodium voltage-gated channels concentrated in the junctional folds and around the NMJ. In our experiment, the ChRs were homogeneously distributed along the membrane and might have generated greater currents than necessary to engage the Nav1.4 to spread the depolarization. Targeting the ChR to the NMJ in a mechanism similar to ACh receptors may amplify the depolarization effect as postulated for ACh receptors (Martin, 1994). With smaller photocurrents necessary for muscle depolarization, lower light intensity could be used lessening desensitization and increasing the versatility of this system for clinical translation.

Conclusion

We have shown that transplantation of ESCMNs in the peripheral nervous system can restore motor function after prolonged denervation as long as ESCMNs are transplanted within a critical time window. We encountered the development of teratocarcinomas generated from pluripotent cells persisting among the transplanted cell population despite neural differentiation. We were successful in eliminating these cells with mitomycin C prior to transplantation. Transplanted ESCMNs integrated into a self-organized circuit with interneurons generated during the stem cell differentiation protocol. This circuit driven by glutamate but under the influence of other neurotransmitters resulted in uncontrolled muscle contractions. These unexpected findings raise questions and further investigations in the stem cell differentiation process and embryonic neuronal network elaboration before this technology gets incorporated in clinical trials. The excision of tumours from transplanted stem cell-derived tissue in the spinal cord or the brain would be surgically dreadful and could be scientifically preventable. We showed that muscle controlled by light pulse after the expression of channelrhodopsin-2 along the sarcolemma. The amount of control over contraction provided by this optogenetic system is extensive and clinically promising. Further developments on delivery vectors, cell expression and membrane targeting of this channel in muscles will most likely deliver this technology to clinical use in the near future.

References

- Abematsu M, Tsujimura K, Yamano M, Saito M, Kohno K, Kohyama J, Namihira M, Komiya S, Nakashima K (2010) Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. *J Clin Invest* 120:3255–3266.
- Altman J, Bayer SA (1984) The development of the rat spinal cord. *Adv Anat Embryol Cell Biol* 85:1–164.
- Andrews PW, Matin MM, Bahrami AR, Damjanov I, Gokhale P, Draper JS (2005) Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin. *Biochem Soc Trans* 33:1526–1530.
- Aravamudan B, Mantilla CB, Zhan W-Z, Sieck GC (2006) Denervation effects on myonuclear domain size of rat diaphragm fibers. *J Appl Physiol* 100:1617–1622.
- Araya R, Riquelme MA, Brandan E, Sáez JC (2004) The formation of skeletal muscle myotubes requires functional membrane receptors activated by extracellular ATP. *Brain Res Brain Res Rev* 47:174–188.
- Argadine HM, Hellyer NJ, Mantilla CB, Zhan WZ, Sieck GC (2009) The effect of denervation on protein synthesis and degradation in adult rat diaphragm muscle. *J Appl Physiol* 107:438–444.
- Armstrong DL, Turin L, Warner AE (1983) Muscle activity and the loss of electrical coupling between striated muscle cells in *Xenopus* embryos. *J Neurosci* 3:1414–1421.
- Bain JR, Veltri KL, Chamberlain D, Fahnestock M (2001) Improved functional recovery of denervated skeletal muscle after temporary sensory nerve innervation. *NSC* 103:503–510.
- Baker DEC, Harrison NJ, Maltby E, Smith K, Moore HD, Shaw PJ, Heath PR, Holden H, Andrews PW (2007) Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nat Biotechnol* 25:207–215.
- Belluardo N, Trovato-Salinaro A, Mudò G, Condorelli DF (2005) Expression of the rat connexin 39 (rCx39) gene in myoblasts and myotubes in developing and regenerating skeletal muscles: an in situ hybridization study. *Cell Tissue Res* 320:299–310.
- Berchtold MW, Brinkmeier H, Müntener M (2000) Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol Rev* 80:1215–1265.
- Bieberich E, Silva J, Wang G, Krishnamurthy K, Condie BG (2004) Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants. *J Cell Biol* 167:723–734.
- Blum B, Bar-Nur O, Golan-Lev T, Benvenisty N (2009) The anti-apoptotic gene survivin contributes to teratoma formation by human embryonic stem cells. *Nat Biotechnol* 27:281–287.
- Blum B, Benvenisty N (2008) The tumorigenicity of human embryonic stem cells. *Adv Cancer Res* 100:133–158.

- Blum B, Benvenisty N (2009) The tumorigenicity of diploid and aneuploid human pluripotent stem cells. *Cell Cycle* 8:3822–3830.
- Bradley WG, Good P, Rasool CG, Adelman LS (1983) Morphometric and biochemical studies of peripheral nerves in amyotrophic lateral sclerosis. *Ann Neurol* 14:267–277.
- Brederlau A, Correia AS, Anisimov SV, Elmi M, Paul G, Roybon L, Morizane A, Bergquist F, Riebe I, Nannmark U, Carta M, Hanse E, Takahashi J, Sasai Y, Funa K, Brundin P, Eriksson PS, Li J-Y (2006) Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation. *Stem Cells* 24:1433–1440.
- Bregman B, Coumans J, Dai H, Kuhn P (2002) Transplants and neurotrophic factors increase regeneration and recovery of function after spinal cord injury. *Progress in brain ...*
- Bretzner F, Gilbert F, Baylis F, Brownstone RM (2011) Target populations for first-in-human embryonic stem cell research in spinal cord injury. *Cell Stem Cell* 8:468–475.
- Buehr M, Nichols J, Stenhouse F, Mountford P, Greenhalgh CJ, Kantachavesiri S, Brooker G, Mullins J, Smith AG (2003) Rapid loss of Oct-4 and pluripotency in cultured rodent blastocysts and derivative cell lines. *Biol Reprod* 68:222–229.
- Burns AS, Jawaid S, Zhong H, Yoshihara H, Bhagat S, Murray M, Roy RR, Tessler A, Son Y-J (2007) Paralysis elicited by spinal cord injury evokes selective disassembly of neuromuscular synapses with and without terminal sprouting in ankle flexors of the adult rat. *J Comp Neurol* 500:116–133.
- Canty AJ, Murphy M (2008) Molecular mechanisms of axon guidance in the developing corticospinal tract. *Prog Neurobiol* 85:214–235.
- Cao Q-L, Zhang YP, Howard RM, Walters WM, Tsoulfas P, Whitemore SR (2001) Pluripotent Stem Cells Engrafted into the Normal or Lesioned Adult Rat Spinal Cord Are Restricted to a Glial Lineage. *Exp Neurol* 167:48–58.
- Carraro U, Boncompagni S, Gobbo V, Rossini K, Zampieri S, Mosole S, Ravara B, Nori A, Stramare R, Ambrosio F, Piccione F, Masiero S, Vindigni V, Gargiulo P, Protasi F, Kern H, Pond A, Marcante A (2015) Persistent muscle fiber regeneration in long term denervation. Past, present, future. *Eur J Transl Myol* 25:77–92.
- Castro MJ, Apple DF, Staron RS, Campos GE, Dudley GA (1999) Influence of complete spinal cord injury on skeletal muscle within 6 mo of injury. *J Appl Physiol* 86:350–358.
- Cea LA, Cisterna BA, Puebla C, Frank M, Figueroa XF, Cardozo C, Willecke K, Latorre R, Sáez JC (2013) De novo expression of connexin hemichannels in denervated fast skeletal muscles leads to atrophy. *Proc Natl Acad Sci USA* 110:16229–16234.
- Celichowski J, Mrówczyński W, Krutki P, Górska T, Majczynski H, Sławińska U (2006) Changes in contractile properties of motor units of the rat medial gastrocnemius muscle after spinal cord transection. *Experimental Physiology* 91:887–895.

Choo AB, Tan HL, Ang SN, Fong WJ, Chin A, Lo J, Zheng L, Hentze H, Philp RJ, Oh SKW, Yap M (2008) Selection against undifferentiated human embryonic stem cells by a cytotoxic antibody recognizing podocalyxin-like protein-1. *Stem Cells* 26:1454–1463.

Clowry G, Sieradzan K, Vrbová G (1991) Transplants of embryonic motoneurons to adult spinal cord: survival and innervation abilities. *Trends Neurosci* 14:355–357.

Clowry GJ, Sieradzan K, Vrbová G (1994) Expression of cholinergic phenotype by embryonic ventral horn neurons transplanted into the spinal cord in the rat. *Restor Neurol Neurosci* 6:209–219.

Collazos-Castro JE, Soto VM, Gutiérrez-Dávila M, Nieto-Sampedro M (2005) Motoneuron loss associated with chronic locomotion impairments after spinal cord contusion in the rat. *J Neurotrauma* 22:544–558.

Constantin B, Cronier L (2000) Involvement of gap junctional communication in myogenesis. *Int Rev Cytol* 196:1–65.

Corti S, Nizzardo M, Nardini M, Donadoni C, Salani S, Ronchi D, Simone C, Falcone M, Papadimitriou D, Locatelli F, Mezzina N, Gianni F, Bresolin N, Comi GP (2010) Embryonic stem cell-derived neural stem cells improve spinal muscular atrophy phenotype in mice. *Brain* 133:465–481.

Coumans JV, Lin TT, Dai HN, MacArthur L, McAtee M, Nash C, Bregman BS (2001) Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. *Journal of Neuroscience* 21:9334–9344.

Cusimano M, Biziato D, Brambilla E, Donega M, Alfaro-Cervello C, Snider S, Salani G, Pucci F, Comi G, Garcia-Verdugo JM, De Palma M, Martino G, Pluchino S (2012) Transplanted neural stem/precursor cells instruct phagocytes and reduce secondary tissue damage in the injured spinal cord. *Brain* 135:447–460.

Damjanov I (1993) Teratocarcinoma: neoplastic lessons about normal embryogenesis. *Int J Dev Biol* 37:39–46.

De Miguel MP, Arnalich Montiel F, Lopez Iglesias P, Blazquez Martinez A, Nistal M (2009) Epiblast-derived stem cells in embryonic and adult tissues. *Int J Dev Biol* 53:1529–1540.

deLapeyrière O, Henderson CE (1997) Motoneuron differentiation, survival and synaptogenesis. *Curr Opin Genet Dev* 7:642–650.

Deshpande DM, Kim Y-S, Martinez T, Carmen J, Dike S, Shats I, Rubin LL, Drummond J, Krishnan C, Hoke A, Maragakis N, Shefner J, Rothstein JD, Kerr DA (2006) Recovery from paralysis in adult rats using embryonic stem cells. *Ann Neurol* 60:32–44.

Dudley-Javoroski S, Shields RK (2008) Muscle and bone plasticity after spinal cord injury: review of adaptations to disuse and to electrical muscle stimulation. *J Rehabil Res Dev* 45:283–296.

Dupont-Versteegden EE (2005) Apoptosis in muscle atrophy: Relevance to sarcopenia. *Experimental Gerontology* 40:473–481.

- Durcova-Hills G, Wianny F, Merriman J, Zernicka-Goetz M, McLaren A (2003) Developmental fate of embryonic germ cells (EGCs), in vivo and in vitro. *Differentiation* 71:135–141.
- Dutt V, Gupta S, Dabur R, Injeti E, Mittal A (2015) Skeletal muscle atrophy: Potential therapeutic agents and their mechanisms of action. *Pharmacological Research* 99:86–100.
- Extavour CG, Akam M (2003) Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development* 130:5869–5884.
- Finkelstein DI, Bartlett PF, Horne MK, Cheema SS (1996) Leukemia inhibitory factor is a myotrophic and neurotrophic agent that enhances the reinnervation of muscle in the rat. *J Neurosci Res* 46:122–128.
- Fu SY, Gordon T (1995a) Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. *J Neurosci* 15:3886–3895.
- Fu SY, Gordon T (1995b) Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy. *J Neurosci* 15:3876–3885.
- Fujikawa T, Oh S-H, Pi L, Hatch HM, Shupe T, Petersen BE (2005) Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am J Pathol* 166:1781–1791.
- Gallo G (2006) RhoA-kinase coordinates F-actin organization and myosin II activity during semaphorin-3A-induced axon retraction. *Journal of Cell Science* 119:3413–3423.
- Gan LS, Prochazka A, Bornes TD, Denington AA, Chan KM (2007) A new means of transcutaneous coupling for neural prostheses. *IEEE transactions on bio-medical engineering* 54:509–517.
- Geens M, Van de Velde H, De Block G, Goossens E, Van Steirteghem A, Tournaye H (2006) The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. *Hum Reprod* 22:733–742.
- Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ (2004) Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 427:148–154.
- Giuffre JL, Kakar S, Bishop AT, Spinner RJ, Shin AY (2010) Current Concepts of the Treatment of Adult Brachial Plexus Injuries. *J Hand Surg Am* 35:678–688.
- Glass DJ (2005) Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* 37:1974–1984.
- Gordon T, Fu SY (1997) Long-term response to nerve injury. *Adv Neurol* 72:185–199.
- Grumbles RM, Almeida VW, Casella GTB, Wood PM, Hemstapat K, Thomas CK (2012) Motoneuron replacement for reinnervation of skeletal muscle in adult rats. *J Neuropathol Exp Neurol* 71:921–930.
- Grumbles RM, Casella GTB, Rudinsky MJ, Godfrey S, Wood PM, Thomas CK (2005) The immunophilin ligand FK506, but not the P38 kinase inhibitor SB203580, improves function of adult rat muscle reinnervated from transplants of embryonic neurons. *NSC* 130:619–630.

Grumbles RM, Wood P, Rudinsky M, Gomez AM, Thomas CK (2002) Muscle reinnervation with delayed or immediate transplant of embryonic ventral spinal cord cells into adult rat peripheral nerve. *Cell transplantation* 11:241–250.

Gulino R, Cataudella T, Casamenti F, Pepeu G, Stanzani S, Leanza G (2007) Acetylcholine release from fetal tissue homotopically grafted to the motoneuron-depleted lumbar spinal cord. An in vivo microdialysis study in the awake rat. *Exp Neurol* 204:326–338.

Hamburger V, Oppenheim RW (1990) Naturally Occurring Neuronal Death in Vertebrates. In: *Neuroembryology*, pp 126–142. Boston, MA: Birkhäuser Boston.

Hanson MG, Landmesser LT (2004) Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules. *Neuron* 43:687–701.

Hermann BP, Sukhwani M, Salati J, Sheng Y, Chu T, Orwig KE (2011) Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. *Hum Reprod* 26:3222–3231.

Hollyday M, Hamburger V (1976) Reduction of the naturally occurring motor neuron loss by enlargement of the periphery. *J Comp Neurol* 170:311–320.

Horvat JC, Baillet-Derbin C, Ye JH, Rhrich F, Affane F (1991) Co-transplantation of embryonic neural tissue and autologous peripheral nerve segments to severe spinal cord injury of the adult rat. Guided axogenesis from transplanted neurons. *Restor Neurol Neurosci* 2:289–298.

Höke A, Gordon T, Zochodne DW, Sulaiman OAR (2002) A decline in glial cell-line-derived neurotrophic factor expression is associated with impaired regeneration after long-term Schwann cell denervation. *Exp Neurol* 173:77–85.

Hyatt J-PK, Roy RR, Baldwin KM, Edgerton VR (2003) Nerve activity-independent regulation of skeletal muscle atrophy: role of MyoD and myogenin in satellite cells and myonuclei. *Am J Physiol, Cell Physiol* 285:C1161–C1173.

Irintchev A, Draguhn A, Wernig A (1990) Reinnervation and recovery of mouse soleus muscle after long-term denervation. *Neuroscience* 39:231–243.

Isaacs J (2010) Treatment of Acute Peripheral Nerve Injuries: Current Concepts. *J Hand Surg Am* 35:491–497.

Jackson CE, Bryan WW (1998) Amyotrophic lateral sclerosis. *Semin Neurol* 18:27–39.

Karimi-Abdolrezaee S, Eftekharpour E, Wang J, Schut D, Fehlings MG (2010) Synergistic Effects of Transplanted Adult Neural Stem/Progenitor Cells, Chondroitinase, and Growth Factors Promote Functional Repair and Plasticity of the Chronically Injured Spinal Cord. *J Neurosci* 30:1657–1676.

Kleinlogel S, Feldbauer K, Dempski RE, Fotis H, Wood PG, Bamann C, Bamberg E (2011) Ultra light-sensitive and fast neuronal activation with the Ca²⁺-permeable channelrhodopsin CatCh. *Nat Neurosci* 14:513–518.

- Kleinsmith LJ, Pierce GB (1964) Multipotentiality of single embryonal carcinoma cells. *Cancer Res* 24:1544–1551.
- Kobayashi J, Mackinnon SE, Watanabe O, Ball DJ, Gu XM, Hunter DA, Kuzon WM (1997) The effect of duration of muscle denervation on functional recovery in the rat model. *Muscle Nerve* 20:858–866.
- Lawrenz B, Schiller H, Willbold E, Ruediger M, Muhs A, Esser S (2004) Highly sensitive biosafety model for stem-cell-derived grafts. *Cytotherapy* 6:212–222.
- Lawson KA, Hage WJ (1994) Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Found Symp* 182:68–84–discussion84–91.
- Leor J, Gerecht S, Cohen S, Miller L, Holbova R, Ziskind A, Shachar M, Feinberg MS, Guetta E, Itskovitz-Eldor J (2007) Human embryonic stem cell transplantation to repair the infarcted myocardium. *Heart* 93:1278–1284.
- Lepore AC, Fischer I (2005) Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. *Exp Neurol* 194:230–242.
- Lin JY, Lin MZ, Steinbach P, Tsien RY (2009) Characterization of engineered channelrhodopsin variants with improved properties and kinetics. *Biophysical Journal* 96:1803–1814.
- Lin S, Xu L, Hu S, Zhang C, Wang Y, Xu J (2013) Optimal time-point for neural stem cell transplantation to delay denervated skeletal muscle atrophy. *Muscle Nerve* 47:194–201.
- Lowrie MB, Vrbová G (1992) Dependence of postnatal motoneurons on their targets: review and hypothesis. *Trends Neurosci* 15:80–84.
- Lowry N, Goderie SK, Adamo M, Lederman P, Charniga C, Gill J, Silver J, Temple S (2008) Multipotent embryonic spinal cord stem cells expanded by endothelial factors and Shh/RA promote functional recovery after spinal cord injury. *Exp Neurol* 209:510–522.
- López-González R, Kunckles P, Velasco I (2009) Transient recovery in a rat model of familial amyotrophic lateral sclerosis after transplantation of motor neurons derived from mouse embryonic stem cells. *Cell transplantation* 18:1171–1181.
- Lu P, Jones L, Tuszynski M (2007) Axon regeneration through scars and into sites of chronic spinal cord injury. *Exp Neurol* 203:8–21.
- Lu P, Jones LL, Snyder EY, Tuszynski MH (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Exp Neurol* 181:115–129.
- Lu P, Wang Y, Graham L, McHale K, Gao M, Di Wu, Brock J, Blesch A, Rosenzweig ES, Havton LA, Zheng B, Conner JM, Marsala M, Tuszynski MH (2012) Long-Distance Growth and Connectivity of Neural Stem Cells after Severe Spinal Cord Injury. *Cell* 150:1264–1273.
- Luo L, O'Leary DDM (2005) Axon retraction and degeneration in development and disease. *Annu Rev Neurosci* 28:127–156.

Ma CHE, Omura T, Cobos EJ, Latremoliere A, Ghasemlou N, Brenner GJ, van Veen E, Barrett L, Sawada T, Gao F, Coppola G, Gertler F, Costigan M, Geschwind D, Woolf CJ (2011) Accelerating axonal growth promotes motor recovery after peripheral nerve injury in mice. *J Clin Invest* 121:4332–4347.

Maltzahn von J, Euwens C, Willecke K, Söhl G (2004) The novel mouse connexin39 gene is expressed in developing striated muscle fibers. *Journal of Cell Science* 117:5381–5392.

Martin AR (1994) Amplification of neuromuscular transmission by postjunctional folds. *Proc Biol Sci* 258:321–326.

Martin LJ, Kaiser A, Price AC (1999) Motor neuron degeneration after sciatic nerve avulsion in adult rat evolves with oxidative stress and is apoptosis. *J Neurobiol* 40:185–201.

McLaren A (2003) Primordial germ cells in the mouse. *Dev Biol* 262:1–15.

Mohajeri MH, Figlewicz DA, Bohn MC (1998) Selective loss of alpha motoneurons innervating the medial gastrocnemius muscle in a mouse model of amyotrophic lateral sclerosis. *Exp Neurol* 150:329–336.

Mrówczyński W, Celichowski J, Krutki P, Cabaj A, Slawinska U, Majczynski H (2011) Changes of the force-frequency relationship in the rat medial gastrocnemius muscle after total transection and hemisection of the spinal cord. *J Neurophysiol* 105:2943–2950.

Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A (2005) Light Activation of Channelrhodopsin-2 in Excitable Cells of *Caenorhabditis elegans* Triggers Rapid Behavioral Responses. *Current Biology* 15:2279–2284.

Nógrádi A, Pajer K, Márton G (2011) The role of embryonic motoneuron transplants to restore the lost motor function of the injured spinal cord. *Ann Anat* 193:362–370.

Nógrádi A, Szabó A (2008) Transplantation of embryonic neurones to replace missing spinal motoneurons. *Restor Neurol Neurosci* 26:215–223.

Nógrádi A, Vrbová G (1996) Improved motor function of denervated rat hindlimb muscles induced by embryonic spinal cord grafts. *Eur J Neurosci* 8:2198–2203.

Ollivier-Lanvin K, Lemay MA, Tessler A, Burns AS (2009) Neuromuscular transmission failure and muscle fatigue in ankle muscles of the adult rat after spinal cord injury. *Journal of Applied Physiology* 107:1190–1194.

Patterson MF, Stephenson GMM, Stephenson DG (2006) Denervation produces different single fiber phenotypes in fast- and slow-twitch hindlimb muscles of the rat. *Am J Physiol, Cell Physiol* 291:C518–C528.

Perry VH, Brown MC (1992) Macrophages and nerve regeneration. *Curr Opin Neurobiol* 2:679–682.

Pfeifer K, Vroemen M, Blesch A, Weidner N (2004) Adult neural progenitor cells provide a permissive guiding substrate for corticospinal axon growth following spinal cord injury. *Eur J Neurosci* 20:1695–1704.

- Qin W, Bauman WA, Cardozo C (2010) Bone and muscle loss after spinal cord injury: organ interactions. *Ann N Y Acad Sci* 1211:66–84.
- Quinlan GA, Williams EA, Tan SS, Tam PP (1995) Neuroectodermal fate of epiblast cells in the distal region of the mouse egg cylinder: implication for body plan organization during early embryogenesis. *Development* 121:87–98.
- Quy PN, Kuma A, Pierre P, Mizushima N (2013) Proteasome-dependent Activation of Mammalian Target of Rapamycin Complex 1 (mTORC1) Is Essential for Autophagy Suppression and Muscle Remodeling Following Denervation. *J Biol Chem* 288:1125–1134.
- Rafuse VF, Gordon T, Orozco R (1992) Proportional enlargement of motor units after partial denervation of cat triceps surae muscles. *J Neurophysiol* 68:1261–1276.
- Ramer LM, Ramer MS, Steeves JD (2005) Setting the stage for functional repair of spinal cord injuries: a cast of thousands. *Spinal Cord* 43:134–161.
- Rassendren FA, Bloch-Gallego E, Tanaka H, Henderson CE (1992) Levels of mRNA coding for motoneuron growth-promoting factors are increased in denervated muscle. *Proc Natl Acad Sci USA* 89:7194–7198.
- Reier PJ (2004) Cellular transplantation strategies for spinal cord injury and translational neurobiology. *NeuroRx* 1:424–451.
- Richardson PM, Issa VMK (1984) Peripheral injury enhances central regeneration of primary sensory neurones. , Published online: 28 June 1984; | doi:101038/309791a0 309:791–793.
- Rossant J, Chazaud C, Yamanaka Y (2003) Lineage allocation and asymmetries in the early mouse embryo. *Philos Trans R Soc Lond, B, Biol Sci* 358:1341–8–discussion1349.
- Ruijs ACJ, Jaquet J-B, Kalmijn S, Giele H, Hovius SER (2005) Median and ulnar nerve injuries: a meta-analysis of predictors of motor and sensory recovery after modern microsurgical nerve repair. *Plast Reconstr Surg* 116:484–94–discussion495–6.
- Rutishauser U, Landmesser L (1991) Polysialic acid on the surface of axons regulates patterns of normal and activity-dependent innervation. *Trends Neurosci* 14:528–532.
- Saito H, Dahlin LB (2008) Expression of ATF3 and axonal outgrowth are impaired after delayed nerve repair. *BMC Neurosci* 9:88.
- Sakuma M, Gorski G, Sheu S-H, Lee S, Barrett LB, Singh B, Omura T, Latremoliere A, Woolf CJ (2015) Lack of motor recovery after prolonged denervation of the neuromuscular junction is not due to regenerative failure Gaspar P, ed. *Eur J Neurosci*:n/a–n/a.
- Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M (2013) Mechanisms regulating skeletal muscle growth and atrophy. *FEBS Journal* 280:4294–4314.
- Shih C-C, Forman SJ, Chu P, Slovak M (2007) Human embryonic stem cells are prone to generate primitive, undifferentiated tumors in engrafted human fetal tissues in severe combined immunodeficient mice. *Stem Cells Dev* 16:893–902.

Sieradzan K, Vrbová G (1989) Replacement of missing motoneurons by embryonic grafts in the rat spinal cord. *NSC* 31:115–130.

Sieradzan K, Vrbová G (1991) Factors influencing survival of transplanted embryonic motoneurons in the spinal cord of adult rats. *Exp Neurol* 114:286–299.

Smith AJ, Nelson NG, Oommen S, Hartjes KA, Folmes CD, Terzic A, Nelson TJ (2012) Apoptotic susceptibility to DNA damage of pluripotent stem cells facilitates pharmacologic purging of teratoma risk. *Stem Cells Transl Med* 1:709–718.

Sortwell CE, Pitzer MR, Collier TJ (2000) Time Course of Apoptotic Cell Death within Mesencephalic Cell Suspension Grafts: Implications for Improving Grafted Dopamine Neuron Survival. *Exp Neurol* 165:268–277.

Spinner RJ, Kline DG (2000) Surgery for peripheral nerve and brachial plexus injuries or other nerve lesions. *Muscle Nerve* 23:680–695.

Squecco R, Carraro U, Kern H, Pond A, Adami N, Biral D, Vindigni V, Boncompagni S, Pietrangelo T, Bosco G, Fanò G, Marini M, Abruzzo PM, Germinario E, Danieli-Betto D, Protasi F, Francini F, Zampieri S (2009) A subpopulation of rat muscle fibers maintains an assessable excitation-contraction coupling mechanism after long-standing denervation despite lost contractility. *J Neuropathol Exp Neurol* 68:1256–1268.

Sulaiman OA, Gordon T (2000) Effects of short- and long-term Schwann cell denervation on peripheral nerve regeneration, myelination, and size. *Glia* 32:234–246.

Sunderland S (1978) *Sunderland: Nerves and injuries* - Google Scholar. Edinburgh: Churchill & Livingstone.

Tam PP, Zhou SX (1996) The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. *Dev Biol* 178:124–132.

Terenghi G (1999) Peripheral nerve regeneration and neurotrophic factors. *J Anat* 194 (Pt 1):1–14.

Theele DP, Schrimsher GW, Reier PJ (1996) Comparison of the growth and fate of fetal spinal iso- and allografts in the adult rat injured spinal cord. *Exp Neurol* 142:128–143.

Thomas CK, Erb DE, Grumbles RM, Bunge RP (2000) Embryonic cord transplants in peripheral nerve restore skeletal muscle function. *J Neurophysiol* 84:591–595.

Thomas CK, Zaidner EY, Calancie B, Broton JG, Bigland-Ritchie BR (1997) Muscle Weakness, Paralysis, and Atrophy after Human Cervical Spinal Cord Injury. *Exp Neurol* 148:414–423.

Tötösy de Zepetnek JE, Zung HV, Erdebil S, Gordon T (1992) Innervation ratio is an important determinant of force in normal and reinnervated rat tibialis anterior muscles. *J Neurophysiol* 67:1385–1403.

Tsang TE, Khoo PL, Jamieson RV, Zhou SX, Ang SL, Behringer R, Tam PP (2001) The allocation and differentiation of mouse primordial germ cells. *Int J Dev Biol* 45:549–555.

- Ung R-V, Rouleau P, Guertin PA (2010) Effects of co-administration of clenbuterol and testosterone propionate on skeletal muscle in paraplegic mice. *J Neurotrauma* 27:1129–1142.
- van Bremen T, Vogt CC, Send T, Fleischmann BK, Bruegmann T, Sasse P (2015) Optogenetic control of contractile function in skeletal muscle. *Nat Comms* 6:1–8.
- Wang H, Sugiyama Y, Hikima T, Sugano E, Tomita H, Takahashi T, Ishizuka T, Yawo H (2009) Molecular determinants differentiating photocurrent properties of two channelrhodopsins from *Chlamydomonas*. *J Biol Chem* 284:5685–5696.
- Wang L, Klein R, Zheng B, Marquardt T (2011) Anatomical Coupling of Sensory and Motor Nerve Trajectory via Axon Tracking. *Neuron* 71:263–277.
- Wen L, Wang H, Tanimoto S, Egawa R, Matsuzaka Y, Mushiake H, Ishizuka T, Yawo H (2010) Opto-current-clamp actuation of cortical neurons using a strategically designed channelrhodopsin. Brezina V, ed. *PLoS ONE* 5:e12893.
- White TE, Lane MA, Sandhu MS, O'Steen BE, Fuller DD, Reier PJ (2010) Neuronal progenitor transplantation and respiratory outcomes following upper cervical spinal cord injury in adult rats. *Exp Neurol* 225:231–236.
- Wong FSY, Chan BP, Lo ACY (2014) Carriers in cell-based therapies for neurological disorders. *Int J Mol Sci* 15:10669–10723.
- Wu Y, Collier L, Pan J, Qin W, Bauman WA, Cardozo CP (2012) Testosterone reduced methylprednisolone-induced muscle atrophy in spinal cord-injured rats. *Spinal Cord* 50:57–62.
- Yang LJ-S, Chang KW-C, Chung KC (2012) A systematic review of nerve transfer and nerve repair for the treatment of adult upper brachial plexus injury. *Neurosurgery* 71:417–29–discussion429.
- Yohn DC, Miles GB, Rafuse VF, Brownstone RM (2008) Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy. *Journal of Neuroscience* 28:12409–12418.
- Zeman RJ, Zhao J, Zhang Y, Zhao W, Wen X, Wu Y, Pan J, Bauman WA, Cardozo C (2009) Differential skeletal muscle gene expression after upper or lower motor neuron transection. *Pflugers Arch - Eur J Physiol* 458:525–535.
- Zhang F, Fischer KA (2002) End-to-side neurorrhaphy. *Microsurgery* 22:122–127.
- Zhang P, Chen X, Fan M (2007) Signaling mechanisms involved in disuse muscle atrophy. *Med Hypotheses* 69:310–321.
- Zhao C, Veltri K, Li S, Bain JR, Fahnestock M (2004) NGF, BDNF, NT-3, and GDNF mRNA expression in rat skeletal muscle following denervation and sensory protection. *J Neurotrauma* 21:1468–1478.
- Zwaka TP, Thomson JA (2005) A germ cell origin of embryonic stem cells? *Development* 132:227–233.

Appendix One

Chapter Three Movie – Transcutaneous Optical Activation Of Muscle Contraction

Transcutaneous tricep surae muscle contractions evoked by single and repetitive pulses of light emitted from a 470 nm blue LED in a Sim1-cre: Ai32 mouse under anesthesia. Tricep surae contractions can be visualized upon light emission. Sustained tetanic contraction can be seen until the light is turned off when the muscle relaxes.

Movie available on the Dalhousie University DalSpace website: <https://dalspace.library.dal.ca>

Appendix Two

Chapter Four Movie – Spontaneous Muscle Contractions Induced From Transplanted ESCMNs In The Tibial Nerve

Transplanted tibial nerve maintained *ex vivo*, viewed under the surgical microscope.

Enlargement at the end of nerve represents the transplantation site. Top muscle is the MG spontaneously contracting. Femur is anchored on the left with pins. Suction electrode on top of muscle for EMG recording. Stimulating electrode at the bottom is not contacting the nerve and not stimulating.

Movie available on the Dalhousie University DalSpace website: <https://dalspace.library.dal.ca>

References

- Abematsu M, Tsujimura K, Yamano M, Saito M, Kohno K, Kohyama J, Namihira M, Komiya S, Nakashima K (2010) Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. *J Clin Invest* 120:3255–3266.
- Ahn H, Bailey CS, Rivers CS, Noonan VK, Tsai EC, Fournay DR, Attabib N, Kwon BK, Christie SD, Fehlings MG, Finkelstein J, Hurlbert RJ, Townson A, Parent S, Drew B, Chen J, Dvorak MF, Rick Hansen Spinal Cord Injury Registry Network (2015) Effect of older age on treatment decisions and outcomes among patients with traumatic spinal cord injury. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne* 187:873–880.
- Al-Majed AA, Neumann CM, Brushart TM, Gordon T (2000) Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. *J Neurosci* 20:2602–2608.
- Altman J, Bayer SA (1984) The development of the rat spinal cord. *Adv Anat Embryol Cell Biol* 85:1–164.
- Anderson KD (2004) Targeting recovery: priorities of the spinal cord-injured population. *J Neurotrauma* 21:1371–1383.
- Andrews PW, Matin MM, Bahrami AR, Damjanov I, Gokhale P, Draper JS (2005) Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin. *Biochem Soc Trans* 33:1526–1530.
- Aravamudan B, Mantilla CB, Zhan W-Z, Sieck GC (2006) Denervation effects on myonuclear domain size of rat diaphragm fibers. *J Appl Physiol* 100:1617–1622.
- Araya R, Riquelme MA, Brandan E, Sáez JC (2004) The formation of skeletal muscle myotubes requires functional membrane receptors activated by extracellular ATP. *Brain Res Brain Res Rev* 47:174–188.
- Arber S, Han B, Mendelsohn M, Smith M, Jessell TM, Sockanathan S (1999) Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. *Neuron* 23:659–674.
- Argadine HM, Hellyer NJ, Mantilla CB, Zhan WZ, Sieck GC (2009) The effect of denervation on protein synthesis and degradation in adult rat diaphragm muscle. *J Appl Physiol* 107:438–444.
- Armstrong DL, Turin L, Warner AE (1983) Muscle activity and the loss of electrical coupling between striated muscle cells in *Xenopus* embryos. *J Neurosci* 3:1414–1421.
- Arnold FJL, Hofmann F, Bengtson CP, Wittmann M, Vanhoutte P, Bading H (2005) Microelectrode array recordings of cultured hippocampal networks reveal a simple model for transcription and protein synthesis-dependent plasticity. *J Physiol (Lond)* 564:3–19.
- Asplund M, Nilsson M, Jacobsson A, Holst von H (2009) Incidence of Traumatic Peripheral Nerve Injuries and Amputations in Sweden between 1998 and 2006. *Neuroepidemiology* 32:217–228.

- Bachoud-Lévi AC et al. (2000) Motor and cognitive improvements in patients with Huntington's disease after neural transplantation. *The Lancet* 356:1975–1979.
- Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI (1995) Embryonic stem cells express neuronal properties in vitro. *Dev Biol* 168:342–357.
- Bain JR, Veltri KL, Chamberlain D, Fahnstock M (2001) Improved functional recovery of denervated skeletal muscle after temporary sensory nerve innervation. *NSC* 103:503–510.
- Baker DEC, Harrison NJ, Maltby E, Smith K, Moore HD, Shaw PJ, Heath PR, Holden H, Andrews PW (2007) Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nat Biotechnol* 25:207–215.
- Baldi JC, Jackson RD, Moraille R, Mysiw WJ (1998) Muscle atrophy is prevented in patients with acute spinal cord injury using functional electrical stimulation. *Spinal Cord* 36:463–469.
- Ban J, Bonifazi P, Pinato G, Broccard FD, Studer L, Torre V, Ruaro ME (2007) Embryonic stem cell-derived neurons form functional networks in vitro. *Stem Cells* 25:738–749.
- Banks GB, Noakes PG (2002) Elucidating the molecular mechanisms that underlie the target control of motoneuron death. *Int J Dev Biol* 46:551–558.
- Barberi T, Klivenyi P, Calingasan NY, Lee H, Kawamata H, Loonam K, Perrier AL, Bruses J, Rubio ME, Topf N, Tabar V, Harrison NL, Beal MF, Moore MAS, Studer L (2003) Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat Biotechnol* 21:1200–1207.
- Barohn RJ, Amato AA (2013) Pattern-recognition approach to neuropathy and neuronopathy. *Neurologic clinics* 31:343–361.
- Bellingham MC (2011) A review of the neural mechanisms of action and clinical efficiency of riluzole in treating amyotrophic lateral sclerosis: what have we learned in the last decade? *CNS Neurosci Ther* 17:4–31.
- Belluardo N, Trovato-Salinaro A, Mudò G, Condorelli DF (2005) Expression of the rat connexin 39 (rCx39) gene in myoblasts and myotubes in developing and regenerating skeletal muscles: an in situ hybridization study. *Cell Tissue Res* 320:299–310.
- Benninger F, Beck H, Wernig M, Tucker KL, Brüstle O, Scheffler B (2003) Functional integration of embryonic stem cell-derived neurons in hippocampal slice cultures. *Journal of Neuroscience* 23:7075–7083.
- Berchtold MW, Brinkmeier H, Müntener M (2000) Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol Rev* 80:1215–1265.
- Bernstein JJ, Tang Y (1984) Structure and function of fetal cortex implanted into degenerating peripheral nerve of adult rat. *Brain Res* 324:243–251.
- Bibel M, Richter J, Schrenk K, Tucker KL, Staiger V, Korte M, Goetz M, Barde Y-A (2004) Differentiation of mouse embryonic stem cells into a defined neuronal lineage. *Nat Neurosci* 7:1003–1009.

- Bieberich E, Silva J, Wang G, Krishnamurthy K, Condie BG (2004) Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants. *J Cell Biol* 167:723–734.
- Blankenship AG, Feller MB (2009) Mechanisms underlying spontaneous patterned activity in developing neural circuits. *Nat Rev Neurosci* 11:18–29.
- Blum B, Bar-Nur O, Golan-Lev T, Benvenisty N (2009) The anti-apoptotic gene survivin contributes to teratoma formation by human embryonic stem cells. *Nat Biotechnol* 27:281–287.
- Blum B, Benvenisty N (2008) The tumorigenicity of human embryonic stem cells. *Adv Cancer Res* 100:133–158.
- Blum B, Benvenisty N (2009) The tumorigenicity of diploid and aneuploid human pluripotent stem cells. *Cell Cycle* 8:3822–3830.
- Boakye M, Leigh BC, Skelly AC (2012) Quality of life in persons with spinal cord injury: comparisons with other populations. *J Neurosurg Spine* 17:29–37.
- Boyd JG, Gordon T (2003) Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons in vivo. *Exp Neurol* 183:610–619.
- Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (2005) Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci* 8:1263–1268.
- Bradley WG, Good P, Rasool CG, Adelman LS (1983) Morphometric and biochemical studies of peripheral nerves in amyotrophic lateral sclerosis. *Ann Neurol* 14:267–277.
- Branchereau P, Chapron J, Meyrand P (2002) Descending 5-hydroxytryptamine raphe inputs repress the expression of serotonergic neurons and slow the maturation of inhibitory systems in mouse embryonic spinal cord. *Journal of Neuroscience* 22:2598–2606.
- Brederlau A, Correia AS, Anisimov SV, Elmi M, Paul G, Roybon L, Morizane A, Bergquist F, Riebe I, Nannmark U, Carta M, Hanse E, Takahashi J, Sasai Y, Funa K, Brundin P, Eriksson PS, Li J-Y (2006) Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation. *Stem Cells* 24:1433–1440.
- Bregman B, Coumans J, Dai H, Kuhn P (2002) Transplants and neurotrophic factors increase regeneration and recovery of function after spinal cord injury. *Progress in brain ...*
- Bretzner F, Gilbert F, Baylis F, Brownstone RM (2011) Target populations for first-in-human embryonic stem cell research in spinal cord injury. *Cell Stem Cell* 8:468–475.
- Bromberg MB (2008) Quality of life in amyotrophic lateral sclerosis. *Phys Med Rehabil Clin N Am* 19:591–605–x–xi.
- Bruegmann T, Malan D, Hesse M, Beiert T, Fuegeman CJ, Fleischmann BK, Sasse P (2010) Optogenetic control of heart muscle in vitro and in vivo. *Nat Meth* 7:897–900.

- Brundin P, Nilsson OG, Strecker RE, Lindvall O, Astedt B, Björklund A (1986) Behavioural effects of human fetal dopamine neurons grafted in a rat model of Parkinson's disease. *Experimental brain research Experimentelle Hirnforschung Expérimentation cérébrale* 65:235–240.
- Bryson JB, Machado CB, Crossley M, Stevenson D, Bros-Facer V, Burrone J, Greensmith L, Lieberam I (2014) Optical Control of Muscle Function by Transplantation of Stem Cell-Derived Motor Neurons in Mice. *Science* 344:94–97.
- Buchthal F, Kühl V (1979) Nerve conduction, tactile sensibility, and the electromyogram after suture or compression of peripheral nerve: a longitudinal study in man. *J Neurol Neurosurg Psychiatr* 42:436–451.
- Buehr M, Nichols J, Stenhouse F, Mountford P, Greenhalgh CJ, Kantachuvesiri S, Brooker G, Mullins J, Smith AG (2003) Rapid loss of Oct-4 and pluripotency in cultured rodent blastocysts and derivative cell lines. *Biol Reprod* 68:222–229.
- Burns AS, Jawaid S, Zhong H, Yoshihara H, Bhagat S, Murray M, Roy RR, Tessler A, Son Y-J (2007) Paralysis elicited by spinal cord injury evokes selective disassembly of neuromuscular synapses with and without terminal sprouting in ankle flexors of the adult rat. *J Comp Neurol* 500:116–133.
- Cajal SY (1959) Degeneration & regeneration of the nervous system.
- Calancie B, Molano MR, Broton JG (2004) EMG for assessing the recovery of voluntary movement after acute spinal cord injury in man. *Clinical Neurophysiology* 115:1748–1759.
- Campbell WW (2008) Evaluation and management of peripheral nerve injury. *Clinical Neurophysiology* 119:1951–1965.
- Canty AJ, Murphy M (2008) Molecular mechanisms of axon guidance in the developing corticospinal tract. *Prog Neurobiol* 85:214–235.
- Cao Q-L, Zhang YP, Howard RM, Walters WM, Tsoulfas P, Whittemore SR (2001) Pluripotent Stem Cells Engrafted into the Normal or Lesioned Adult Rat Spinal Cord Are Restricted to a Glial Lineage. *Exp Neurol* 167:48–58.
- Carlstedt T, Cullheim S (2000) Spinal cord motoneuron maintenance, injury and repair. *Prog Brain Res* 127:501–514.
- Carlstedt T, Grane P, Hallin RG, Norén G (1995) Return of function after spinal cord implantation of avulsed spinal nerve roots. *The Lancet* 346:1323–1325.
- Carraro U, Boncompagni S, Gobbo V, Rossini K, Zampieri S, Mosole S, Ravara B, Nori A, Stramare R, Ambrosio F, Piccione F, Masiero S, Vindigni V, Gargiulo P, Protasi F, Kern H, Pond A, Marcante A (2015) Persistent muscle fiber regeneration in long term denervation. Past, present, future. *Eur J Transl Myol* 25:77–92.
- Castro MJ, Apple DF, Staron RS, Campos GE, Dudley GA (1999) Influence of complete spinal cord injury on skeletal muscle within 6 mo of injury. *J Appl Physiol* 86:350–358.

Cea LA, Cisterna BA, Puebla C, Frank M, Figueroa XF, Cardozo C, Willecke K, Latorre R, Sáez JC (2013) De novo expression of connexin hemichannels in denervated fast skeletal muscles leads to atrophy. *Proc Natl Acad Sci USA* 110:16229–16234.

Celichowski J, Mrówczyński W, Krutki P, Górska T, Majczynski H, Sławińska U (2006) Changes in contractile properties of motor units of the rat medial gastrocnemius muscle after spinal cord transection. *Experimental Physiology* 91:887–895.

Chancellor AM, Slattery JM, Fraser H, Swingler RJ, Holloway SM, Warlow CP (1993) The prognosis of adult-onset motor neuron disease: a prospective study based on the Scottish Motor Neuron Disease Register. *Journal of Neurology* 240:339–346.

Choi PD, Novak CB, Mackinnon SE, Kline DG (1997) Quality of life and functional outcome following brachial plexus injury. *J Hand Surg Am* 22:605–612.

Choo AB, Tan HL, Ang SN, Fong WJ, Chin A, Lo J, Zheng L, Hentze H, Philp RJ, Oh SKW, Yap M (2008) Selection against undifferentiated human embryonic stem cells by a cytotoxic antibody recognizing podocalyxin-like protein-1. *Stem Cells* 26:1454–1463.

Christopherson KS, Ullian EM, Stokes CCA, Mullen CE, Hell JW, Agah A, Lawler J, Moshier DF, Bornstein P, Barres BA (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120:421–433.

Chung S, Shin B-S, Hedlund E, Pruszak J, Ferree A, Kang UJ, Isacson O, Kim K-S (2006) Genetic selection of sox1GFP-expressing neural precursors removes residual tumorigenic pluripotent stem cells and attenuates tumor formation after transplantation. *J Neurochem* 97:1467–1480.

Cisterna BA, Cardozo C, Sáez JC (2014) Neuronal involvement in muscular atrophy. *Front Cell Neurosci* 8:405.

Clowry G, Sieradzan K, Vrbová G (1991) Transplants of embryonic motoneurons to adult spinal cord: survival and innervation abilities. *Trends Neurosci* 14:355–357.

Clowry GJ, Sieradzan K, Vrbová G (1994) Expression of cholinergic phenotype by embryonic ventral horn neurons transplanted into the spinal cord in the rat. *Restor Neurol Neurosci* 6:209–219.

Collazos-Castro JE, Soto VM, Gutiérrez-Dávila M, Nieto-Sampedro M (2005) Motoneuron loss associated with chronic locomotion impairments after spinal cord contusion in the rat. *J Neurotrauma* 22:544–558.

Constantin B, Cronier L (2000) Involvement of gap junctional communication in myogenesis. *Int Rev Cytol* 196:1–65.

Corti S, Nizzardo M, Nardini M, Donadoni C, Salani S, Ronchi D, Simone C, Falcone M, Papadimitriou D, Locatelli F, Mezzina N, Gianni F, Bresolin N, Comi GP (2010) Embryonic stem cell-derived neural stem cells improve spinal muscular atrophy phenotype in mice. *Brain* 133:465–481.

Coumailleau P, Duprez D (2009) Sim1 and Sim2 expression during chick and mouse limb development. *International Journal of Developmental ...*

- Coumans JV, Lin TT, Dai HN, MacArthur L, McAtee M, Nash C, Bregman BS (2001) Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. *Journal of Neuroscience* 21:9334–9344.
- Cui L, Jiang J, Wei L, Zhou X, Fraser J, Snider B, Yu S (2008) Transplantation of Embryonic Stem Cells Improves Nerve Repair and Functional Recovery after Severe Sciatic Nerve Axotomy in Rats. *Stem Cells*.
- Cunningham JJ, Ulbright TM, Pera MF, Looijenga LHJ (2012) Lessons from human teratomas to guide development of safe stem cell therapies. *Nat Biotechnol* 30:849–857.
- Cusimano M, Biziato D, Brambilla E, Donega M, Alfaro-Cervello C, Snider S, Salani G, Pucci F, Comi G, Garcia-Verdugo JM, De Palma M, Martino G, Pluchino S (2012) Transplanted neural stem/precursor cells instruct phagocytes and reduce secondary tissue damage in the injured spinal cord. *Brain* 135:447–460.
- Czarnecki A, Le Corrionc H, Rigato C, Le Bras B, Couraud F, Scain AL, Allain AE, Mouffle C, Bullier E, Mangin JM, Branchereau P, Legendre P (2014) Acetylcholine Controls GABA-, Glutamate-, and Glycine-Dependent Giant Depolarizing Potentials that Govern Spontaneous Motoneuron Activity at the Onset of Synaptogenesis in the Mouse Embryonic Spinal Cord. *J Neurosci* 34:6389–6404.
- Damjanov I (1993) Teratocarcinoma: neoplastic lessons about normal embryogenesis. *Int J Dev Biol* 37:39–46.
- Dantes M, McComas A (1991) The extent and time course of motoneuron involvement in amyotrophic lateral sclerosis. *Muscle Nerve* 14:416–421.
- de Carvalho M, Dengler R, Eisen A, England JD, Kaji R, Kimura J, Mills K, Mitsumoto H, Nodera H, Shefner J, Swash M (2008) Electrodiagnostic criteria for diagnosis of ALS. *Clinical Neurophysiology* 119:497–503.
- De Miguel MP, Arnalich Montiel F, Lopez Iglesias P, Blazquez Martinez A, Nistal M (2009) Epiblast-derived stem cells in embryonic and adult tissues. *Int J Dev Biol* 53:1529–1540.
- de Zepetnek JET, Gordon T, Stein RB, Zung HV (2011) Comparison of force and EMG measures in normal and reinnervated tibialis anterior muscles of the rat. *Can J Physiol Pharmacol* 69:1774–1783.
- Deardorff AS, Romer SH, Deng Z, Bullinger KL, Nardelli P, Cope TC, Fyffe REW (2012) Expression of postsynaptic Ca²⁺-activated K⁺ (SK) channels at C-bouton synapses in mammalian lumbar-motoneurons. *J Physiol (Lond)*.
- DeJesus-Hernandez M et al. (2011) Expanded GGGGCC Hexanucleotide Repeat in Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS. *Neuron* 72:245–256.
- del Aguila MA, Longstreth WT, McGuire V, Koepsell TD, van Belle G (2003) Prognosis in amyotrophic lateral sclerosis: A population-based study. *Neurology* 60:813–819.
- deLapeyrière O, Henderson CE (1997) Motoneuron differentiation, survival and synaptogenesis. *Curr Opin Genet Dev* 7:642–650.

- Deshpande DM, Kim Y-S, Martinez T, Carmen J, Dike S, Shats I, Rubin LL, Drummond J, Krishnan C, Hoke A, Maragakis N, Shefner J, Rothstein JD, Kerr DA (2006) Recovery from paralysis in adult rats using embryonic stem cells. *Ann Neurol* 60:32–44.
- Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Eggan K (2008) Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons. *Science* 321:1218–1221.
- Dudley-Javoroski S, Shields RK (2008) Muscle and bone plasticity after spinal cord injury: review of adaptations to disuse and to electrical muscle stimulation. *J Rehabil Res Dev* 45:283–296.
- Dunnett SB (1991) Towards a neural transplantation therapy for Parkinson's disease: experimental principles from animal studies. *Acta Neurochir Suppl (Wien)* 52:35–38.
- Dupont-Versteegden EE (2005) Apoptosis in muscle atrophy: Relevance to sarcopenia. *Experimental Gerontology* 40:473–481.
- Durcova-Hills G, Wianny F, Merriman J, Zernicka-Goetz M, McLaren A (2003) Developmental fate of embryonic germ cells (EGCs), in vivo and in vitro. *Differentiation* 71:135–141.
- Dutt V, Gupta S, Dabur R, Injeti E, Mittal A (2015) Skeletal muscle atrophy: Potential therapeutic agents and their mechanisms of action. *Pharmacological Research* 99:86–100.
- Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75:1417–1430.
- Erb DE, Mora RJ, Bunge RP (1993) Reinnervation of adult rat gastrocnemius muscle by embryonic motoneurons transplanted into the axotomized tibial nerve. *Exp Neurol* 124:372–376.
- Evans M (2011) Discovering pluripotency: 30 years of mouse embryonic stem cells. *Nat Rev Mol Cell Biol* 12:680–686.
- Extavour CG, Akam M (2003) Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development* 130:5869–5884.
- Fedorov VV, Lozinsky IT, Sosunov EA, Anyukhovskiy EP, Rosen MR, Balke CW, Efimov IR (2007) Application of blebbistatin as an excitation–contraction uncoupler for electrophysiologic study of rat and rabbit hearts. *Heart Rhythm* 4:619–626.
- Feldman EL, Boulis NM, Hur J, Johe K, Rutkove SB, Federici T, Polak M, Bordeau J, Sakowski SA, Glass JD (2014) Intraspinal neural stem cell transplantation in amyotrophic lateral sclerosis: Phase 1 trial outcomes. *Ann Neurol* 75:363–373.
- Finkelstein DI, Bartlett PF, Horne MK, Cheema SS (1996) Leukemia inhibitory factor is a myotrophic and neurotrophic agent that enhances the reinnervation of muscle in the rat. *J Neurosci Res* 46:122–128.

Fowler TJ, Danta G, Gilliatt RW (1972) Recovery of nerve conduction after a pneumatic tourniquet: observations on the hind-limb of the baboon. *J Neurol Neurosurg Psychiatr* 35:638–647.

Fu SY, Gordon T (1995a) Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. *J Neurosci* 15:3886–3895.

Fu SY, Gordon T (1995b) Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy. *J Neurosci* 15:3876–3885.

Fujikawa T, Oh S-H, Pi L, Hatch HM, Shupe T, Petersen BE (2005) Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am J Pathol* 166:1781–1791.

Furey MJ, Midha R, Xu Q-G, Belkas J, Gordon T (2007) Prolonged Target Deprivation Reduces the Capacity of Injured Motoneurons to Regenerate. 60:723??733.

Furlan JC, Noonan V, Singh A, Fehlings MG (2011) Assessment of impairment in patients with acute traumatic spinal cord injury: a systematic review of the literature. *J Neurotrauma* 28:1445–1477.

Gage FH, Temple S (2013) Neural Stem Cells: Generating and Regenerating the Brain. *Neuron* 80:588–601.

Gallo G (2006) RhoA-kinase coordinates F-actin organization and myosin II activity during semaphorin-3A-induced axon retraction. *Journal of Cell Science* 119:3413–3423.

Gan LS, Prochazka A, Bornes TD, Denington AA, Chan KM (2007) A new means of transcutaneous coupling for neural prostheses. *IEEE transactions on bio-medical engineering* 54:509–517.

Gao BX, Ziskind-Conhaim L (1995) Development of glycine- and GABA-gated currents in rat spinal motoneurons. *J Neurophysiol* 74:113–121.

Geens M, Van de Velde H, De Block G, Goossens E, Van Steirteghem A, Tournaye H (2006) The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. *Hum Reprod* 22:733–742.

Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ (2004) Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 427:148–154.

Giuffre JL, Kakar S, Bishop AT, Spinner RJ, Shin AY (2010) Current Concepts of the Treatment of Adult Brachial Plexus Injuries. *J Hand Surg Am* 35:678–688.

Glass DJ (2005) Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* 37:1974–1984.

Gomez TM, Spitzer NC (1999) In vivo regulation of axon extension and pathfinding by growth-cone calcium transients. *Nature* 397:350–355.

- Gonzalez-Islas C, Garcia-Bereguian MA, O'Flaherty B, Wenner P (2015) Tonic nicotinic transmission enhances spinal GABAergic presynaptic release and the frequency of spontaneous network activity. *Dev Neurobiol*:n/a–n/a.
- Gordon T, Fu SY (1997) Long-term response to nerve injury. *Adv Neurol* 72:185–199.
- Gosgnach S, Lanuza GM, Butt SJB, Saueressig H, Zhang Y, Velasquez T, Riethmacher D, Callaway EM, Kiehn O, Goulding M (2006) V1 spinal neurons regulate the speed of vertebrate locomotor outputs. *Nature* 440:215–219.
- Grossman SD, Rosenberg LJ, Wrathall JR (2001) Temporal-spatial pattern of acute neuronal and glial loss after spinal cord contusion. *Exp Neurol* 168:273–282.
- Grumbles RM, Almeida VW, Casella GTB, Wood PM, Hemstapat K, Thomas CK (2012) Motoneuron replacement for reinnervation of skeletal muscle in adult rats. *J Neuropathol Exp Neurol* 71:921–930.
- Grumbles RM, Casella GTB, Rudinsky MJ, Godfrey S, Wood PM, Thomas CK (2005) The immunophilin ligand FK506, but not the P38 kinase inhibitor SB203580, improves function of adult rat muscle reinnervated from transplants of embryonic neurons. *NSC* 130:619–630.
- Grumbles RM, Sesodia S, Wood PM, Thomas CK (2009) Neurotrophic factors improve motoneuron survival and function of muscle reinnervated by embryonic neurons. *J Neuropathol Exp Neurol* 68:736–746.
- Grumbles RM, Wood P, Rudinsky M, Gomez AM, Thomas CK (2002) Muscle reinnervation with delayed or immediate transplant of embryonic ventral spinal cord cells into adult rat peripheral nerve. *Cell transplantation* 11:241–250.
- Gulino R, Cataudella T, Casamenti F, Pepeu G, Stanzani S, Leanza G (2007) Acetylcholine release from fetal tissue homotopically grafted to the motoneuron-depleted lumbar spinal cord. An in vivo microdialysis study in the awake rat. *Exp Neurol* 204:326–338.
- Gutmann E (1948) Effect of delay of innervation on recovery of muscle after nerve lesions. *J Neurophysiol* 11:279–294.
- Gutmann E, Young JZ (1944) The re-innervation of muscle after various periods of atrophy. *J Anat* 78:15–43.
- Hamburger V, Oppenheim RW (1990) Naturally Occurring Neuronal Death in Vertebrates. In: *Neuroembryology*, pp 126–142. Boston, MA: Birkhäuser Boston.
- Hammond MW, Xydas D, Downes JH, Bucci G, Becerra V, Warwick K, Constanti A, Nasuto SJ, Whalley BJ (2013) Endogenous cholinergic tone modulates spontaneous network level neuronal activity in primary cortical cultures grown on multi-electrode arrays. *BMC Neurosci* 14:38.
- Hanson MG, Landmesser LT (2003) Characterization of the circuits that generate spontaneous episodes of activity in the early embryonic mouse spinal cord. *Journal of Neuroscience* 23:587–600.

- Hanson MG, Landmesser LT (2004) Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules. *Neuron* 43:687–701.
- Hanson MG, Landmesser LT (2006) Increasing the frequency of spontaneous rhythmic activity disrupts pool-specific axon fasciculation and pathfinding of embryonic spinal motoneurons. *J Neurosci* 26:12769–12780.
- Hanson MG, Milner LD, Landmesser LT (2008) Spontaneous rhythmic activity in early chick spinal cord influences distinct motor axon pathfinding decisions. *Brain Research Reviews* 57:77–85.
- Harper JM, Krishnan C, Darman JS, Deshpande DM, Peck S, Shats I, Backovic S, Rothstein JD, Kerr DA (2004) Axonal growth of embryonic stem cell-derived motoneurons in vitro and in motoneuron-injured adult rats. *Proc Natl Acad Sci USA* 101:7123–7128.
- Hegedus J, Putman CT, Tyreman N, Gordon T (2008) Preferential motor unit loss in the SOD1 G93A transgenic mouse model of amyotrophic lateral sclerosis. *J Physiol (Lond)* 586:3337–3351.
- Heikkilä TJ, Ylä-Outinen L, Tanskanen JMA, Lappalainen RS, Skottman H, Suuronen R, Mikkonen JE, Hyttinen JAK, Narkilahti S (2009) Human embryonic stem cell-derived neuronal cells form spontaneously active neuronal networks in vitro. *Exp Neurol* 218:109–116.
- Henneman E, Somjen G (1965) Functional significance of cell size in spinal motoneurons. *Journal of*
- Hentze H, Graichen R, Colman A (2007) Cell therapy and the safety of embryonic stem cell-derived grafts. *Trends in Biotechnology* 25:24–32.
- Hermann BP, Sukhwani M, Salati J, Sheng Y, Chu T, Orwig KE (2011) Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. *Hum Reprod* 26:3222–3231.
- Ho CH, Triolo RJ, Elias AL, Kilgore KL, DiMarco AF, Bogie K, Vette AH, Audu ML, Kobetic R, Chang SR, Chan KM, Dukelow S, Bourbeau DJ, Brose SW, Gustafson KJ, Kiss ZHT, Mushahwar VK (2014) Functional electrical stimulation and spinal cord injury. *Phys Med Rehabil Clin N Am* 25:631–54–ix.
- Hoffman H (1950) Local re-innervation in partially denervated muscle; a histophysiological study. *Aust J Exp Biol Med Sci* 28:383–397.
- Hollyday M, Hamburger V (1976) Reduction of the naturally occurring motor neuron loss by enlargement of the periphery. *J Comp Neurol* 170:311–320.
- Horvat JC, Baillet-Derbin C, Ye JH, Rhrich F, Affane F (1991) Co-transplantation of embryonic neural tissue and autologous peripheral nerve segments to severe spinal cord injury of the adult rat. Guided axogenesis from transplanted neurons. *Restor Neurol Neurosci* 2:289–298.
- Höke A, Gordon T, Zochodne DW, Sulaiman OAR (2002) A decline in glial cell-line-derived neurotrophic factor expression is associated with impaired regeneration after long-term Schwann cell denervation. *Exp Neurol* 173:77–85.

- Huber D, Petreanu L, Ghitani N, Ranade S, Hromádka T, Mainen Z, Svoboda K (2008) Sparse optical microstimulation in barrel cortex drives learned behaviour in freely moving mice. *Nature* 451:61–64.
- Hyatt J-PK, Roy RR, Baldwin KM, Edgerton VR (2003) Nerve activity-independent regulation of skeletal muscle atrophy: role of MyoD and myogenin in satellite cells and myonuclei. *Am J Physiol, Cell Physiol* 285:C1161–C1173.
- Illes S, Jakab M, Beyer F, Gelfert R, Couillard-Despres S, Schnitzler A, Ritter M, Aigner L (2014) Intrinsically Active and Pacemaker Neurons in Pluripotent Stem Cell-Derived Neuronal Populations. *Stem Cell Reports* 2:323–336.
- Irintchev A, Draguhn A, Wernig A (1990) Reinnervation and recovery of mouse soleus muscle after long-term denervation. *Neuroscience* 39:231–243.
- Isaacs J (2010) Treatment of Acute Peripheral Nerve Injuries: Current Concepts. *J Hand Surg Am* 35:491–497.
- Ishizuka T, Kakuda M, Araki R, Yawo H (2006) Kinetic evaluation of photosensitivity in genetically engineered neurons expressing green algae light-gated channels. *Neuroscience Research* 54:85–94.
- Jackson CE, Bryan WW (1998) Amyotrophic lateral sclerosis. *Semin Neurol* 18:27–39.
- Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nature Reviews Genetics* 1:20–29.
- Jiang Z, Rempel J, Li J, Sawchuk MA, Carlin KP, Brownstone RM (1999) Development of L-type calcium channels and a nifedipine-sensitive motor activity in the postnatal mouse spinal cord. *Eur J Neurosci* 11:3481–3487.
- Kang H, Tian L, Mikesh M, Lichtman JW, Thompson WJ (2014) Terminal Schwann cells participate in neuromuscular synapse remodeling during reinnervation following nerve injury. *Journal of Neuroscience* 34:6323–6333.
- Karimi-Abdolrezaee S, Eftekharpour E, Wang J, Schut D, Fehlings MG (2010) Synergistic Effects of Transplanted Adult Neural Stem/Progenitor Cells, Chondroitinase, and Growth Factors Promote Functional Repair and Plasticity of the Chronically Injured Spinal Cord. *J Neurosci* 30:1657–1676.
- Kirkby LA, Sack GS, Firl A, Feller MB (2013) A role for correlated spontaneous activity in the assembly of neural circuits. *Neuron* 80:1129–1144.
- Kiuru M, Boyer JL, O'Connor TP, Crystal RG (2009) Genetic Control of Wayward Pluripotent Stem Cells and Their Progeny after Transplantation. *Stem Cell* 4:289–300.
- Kleinlogel S, Feldbauer K, Dempski RE, Fotis H, Wood PG, Bamann C, Bamberg E (2011) Ultra light-sensitive and fast neuronal activation with the Ca²⁺-permeable channelrhodopsin CatCh. *Nat Neurosci* 14:513–518.
- Kleinsmith LJ, Pierce GB (1964) MULTIPOTENTIALITY OF SINGLE EMBRYONAL CARCINOMA CELLS. *Cancer Res* 24:1544–1551.

Kline DG (1977) Physiological and clinical factors contributing to the timing of nerve repair. *Clin Neurosurg* 24:425–455.

Knoepfler PS (2009) Deconstructing Stem Cell Tumorigenicity: A Roadmap to Safe Regenerative Medicine. *Stem Cells* 27:1050–1056.

Knutsdottir S, Thorisdottir H, Sigvaldason K, Jónsson H, Björnsson A, Ingvarsson P (2012) Epidemiology of traumatic spinal cord injuries in Iceland from 1975 to 2009. *Spinal Cord* 50:123–126.

Kobayashi J, Mackinnon SE, Watanabe O, Ball DJ, Gu XM, Hunter DA, Kuzon WM (1997) The effect of duration of muscle denervation on functional recovery in the rat model. *Muscle Nerve* 20:858–866.

Krueger H, Noonan VK, Trenaman LM, Joshi P, Rivers CS (2013) The economic burden of traumatic spinal cord injury in Canada. *Chronic Dis Inj Can* 33:113–122.

Kulbatski I, Mothe AJ, Keating A, Hakamata Y, Kobayashi E, Tator CH (2007) Oligodendrocytes and radial glia derived from adult rat spinal cord progenitors: morphological and immunocytochemical characterization. *J Histochem Cytochem* 55:209–222.

Lawrenz B, Schiller H, Willbold E, Ruediger M, Muhs A, Esser S (2004) Highly sensitive biosafety model for stem-cell-derived grafts. *Cytotherapy* 6:212–222.

Lawson KA, Hage WJ (1994) Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Found Symp* 182:68–84–discussion84–91.

Lee AS, Tang C, Rao MS, Weissman IL, Wu JC (2013a) Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies. *Nat Med* 19:998–1004.

Lee M-O, Moon SH, Jeong H-C, Yi J-Y, Lee T-H, Shim SH, Rhee Y-H, Lee S-H, Oh S-J, Lee M-Y, Han M-J, Cho YS, Chung H-M, Kim K-S, Cha H-J (2013b) Inhibition of pluripotent stem cell-derived teratoma formation by small molecules. *Proc Natl Acad Sci USA* 110:E3281–E3290.

Lee S-H, Lumelsky N, Studer L, Auerbach JM, McKay RD (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* 18:675–679.

Leor J, Gerecht S, Cohen S, Miller L, Holbova R, Ziskind A, Shachar M, Feinberg MS, Guetta E, Itskovitz-Eldor J (2007) Human embryonic stem cell transplantation to repair the infarcted myocardium. *Heart* 93:1278–1284.

Lepore AC, Fischer I (2005) Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. *Exp Neurol* 194:230–242.

Li H, Terenghi G, Hall SM (1997) Effects of delayed re-innervation on the expression of c-erbB receptors by chronically denervated rat Schwann cells in vivo. *Glia* 20:333–347.

Limouze J, Straight AF, Mitchison T, Sellers JR (2004) Specificity of blebbistatin, an inhibitor of myosin II. *J Muscle Res Cell Motil* 25:337–341.

Lin JY (2012) Optogenetic excitation of neurons with channelrhodopsins: light instrumentation, expression systems, and channelrhodopsin variants. *Prog Brain Res* 196:29–47.

Lin JY, Lin MZ, Steinbach P, Tsien RY (2009) Characterization of engineered channelrhodopsin variants with improved properties and kinetics. *Biophysical Journal* 96:1803–1814.

Lin S, Xu L, Hu S, Zhang C, Wang Y, Xu J (2013) Optimal time-point for neural stem cell transplantation to delay denervated skeletal muscle atrophy. *Muscle Nerve* 47:194–201.

Lindvall O, Brundin P, Widner H, Rehnström S, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, Marsden CD (1990) Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science* 247:574–577.

Llewellyn ME, Thompson KR, Deisseroth K, Delp SL (2010) Orderly recruitment of motor units under optical control in vivo. *Nat Med* 16:1161–1165.

Lowrie MB, Vrbová G (1992) Dependence of postnatal motoneurons on their targets: review and hypothesis. *Trends Neurosci* 15:80–84.

Lowry N, Goderie SK, Adamo M, Lederman P, Charniga C, Gill J, Silver J, Temple S (2008) Multipotent embryonic spinal cord stem cells expanded by endothelial factors and Shh/RA promote functional recovery after spinal cord injury. *Exp Neurol* 209:510–522.

López-González R, Kunckles P, Velasco I (2009) Transient recovery in a rat model of familial amyotrophic lateral sclerosis after transplantation of motor neurons derived from mouse embryonic stem cells. *Cell transplantation* 18:1171–1181.

Lu P, Jones L, Tuszynski M (2007) Axon regeneration through scars and into sites of chronic spinal cord injury. *Exp Neurol* 203:8–21.

Lu P, Jones LL, Snyder EY, Tuszynski MH (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Exp Neurol* 181:115–129.

Lu P, Wang Y, Graham L, McHale K, Gao M, Di Wu, Brock J, Blesch A, Rosenzweig ES, Havton LA, Zheng B, Conner JM, Marsala M, Tuszynski MH (2012) Long-Distance Growth and Connectivity of Neural Stem Cells after Severe Spinal Cord Injury. *Cell* 150:1264–1273.

Lukas J-R, Aigner M, Denk M, Heinzl H, Burian M, Mayr R (n.d.) Carbocyanine Postmortem Neuronal Tracing: Influence of Different Parameters on Tracing Distance and Combination with Immunocytochemistry.

Luo L, O'Leary DDM (2005) Axon retraction and degeneration in development and disease. *Annu Rev Neurosci* 28:127–156.

Ma CHE, Omura T, Cobos EJ, Latremoliere A, Ghasemlou N, Brenner GJ, van Veen E, Barrett L, Sawada T, Gao F, Coppola G, Gertler F, Costigan M, Geschwind D, Woolf CJ (2011) Accelerating axonal growth promotes motor recovery after peripheral nerve injury in mice. *J Clin Invest* 121:4332–4347.

Macdonald WA, Pedersen TH, Clausen T, Nielsen OB (2005) N-Benzyl-p-toluene sulphonamide allows the recording of trains of intracellular action potentials from nerve-stimulated intact fast-twitch skeletal muscle of the rat. *Experimental Physiology* 90:815–825.

- Maden M (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nat Rev Neurosci* 8:755–765.
- Madisen L et al. (2012) A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. *Nat Neurosci* 15:793–802.
- Maltzahn von J, Euwens C, Willecke K, Söhl G (2004) The novel mouse connexin39 gene is expressed in developing striated muscle fibers. *Journal of Cell Science* 117:5381–5392.
- Marder E, Rehm KJ (2005) Development of central pattern generating circuits. *Curr Opin Neurobiol* 15:86–93.
- Martens DJ, Seaberg RM, van der Kooy D (2002) In vivo infusions of exogenous growth factors into the fourth ventricle of the adult mouse brain increase the proliferation of neural progenitors around the fourth ventricle and the central canal of the spinal cord. *Eur J Neurosci* 16:1045–1057.
- Martin AR (1994) Amplification of neuromuscular transmission by postjunctional folds. *Proc Biol Sci* 258:321–326.
- Martin LJ, Kaiser A, Price AC (1999) Motor neuron degeneration after sciatic nerve avulsion in adult rat evolves with oxidative stress and is apoptosis. *J Neurobiol* 40:185–201.
- Martin-Caraballo M, Greer JJ (1999) Electrophysiological properties of rat phrenic motoneurons during perinatal development. *J Neurophysiol* 81:1365–1378.
- Matsuda R, Yoshikawa M, Kimura H, Ouji Y, Nakase H, Nishimura F, Nonaka J-I, Toriumi H, Yamada S, Nishiofuku M, Moriya K, Ishizaka S, Nakamura M, Sakaki T (2009) Cotransplantation of mouse embryonic stem cells and bone marrow stromal cells following spinal cord injury suppresses tumor development. *Cell transplantation* 18:39–54.
- Mccomas AJ, Fawcett PRW, Campbell MJ, Sica REP (1971) Electrophysiological estimation of the number of motor units within a human muscle. *J Neurol Neurosurg Psychiatr* 34:121–131.
- McLaren A (2003) Primordial germ cells in the mouse. *Dev Biol* 262:1–15.
- Metzger F, Wiese S, Sendtner M (1998) Effect of glutamate on dendritic growth in embryonic rat motoneurons. *J Neurosci* 18:1735–1742.
- Miles GB, Yohn DC, Wichterle H, Jessell TM, Rafuse VF, Brownstone RM (2004) Functional properties of motoneurons derived from mouse embryonic stem cells. *Journal of Neuroscience* 24:7848–7858.
- Miller RG, Mitchell JD, Moore DH (2012) Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). Miller RG, ed. *Cochrane Database Syst Rev* 3:CD001447.
- Miller TM et al. (2013) An antisense oligonucleotide against SOD1 delivered intrathecally for patients with SOD1 familial amyotrophic lateral sclerosis: a phase 1, randomised, first-in-man study. *The Lancet Neurology* 12:435–442.
- Millesi H, Meissl G, Berger A (1973) The interfascicular nerve-grafting of median and ulnar nerves. *Plast Reconstr Surg* 51:233.

- Milner Brown HS, Stein RB (1975) The relation between the surface electromyogram and muscular force. *J Physiol (Lond)* 246:549–569.
- Milner Brown HS, Stein RB, Yemm R (1973) Changes in firing rate of human motor units during linearly changing voluntary contractions. *J Physiol (Lond)* 230:371–390.
- Mohajeri MH, Figlewicz DA, Bohn MC (1998) Selective loss of alpha motoneurons innervating the medial gastrocnemius muscle in a mouse model of amyotrophic lateral sclerosis. *Exp Neurol* 150:329–336.
- Moritani T, deVries HA (1979) Neural factors versus hypertrophy in the time course of muscle strength gain. *Am J Phys Med* 58:115–130.
- Mothe AJ, Tator CH (2013) Review of transplantation of neural stem/progenitor cells for spinal cord injury. *Int J Dev Neurosci* 31:701–713.
- Mothe AJ, Zahir T, Santaguida C, Cook D, Tator CH (2011) Neural stem/progenitor cells from the adult human spinal cord are multipotent and self-renewing and differentiate after transplantation. *PLoS ONE* 6:e27079.
- Mrówczyński W, Celichowski J, Krutki P, Cabaj A, Ślawinska U, Majczyński H (2011) Changes of the force-frequency relationship in the rat medial gastrocnemius muscle after total transection and hemisection of the spinal cord. *J Neurophysiol* 105:2943–2950.
- Myers CP, Lewcock JW, Hanson MG, Gosgnach S, Aimone JB, Gage FH, Lee K-F, Landmesser LT, Pfaff SL (2005) Cholinergic input is required during embryonic development to mediate proper assembly of spinal locomotor circuits. *Neuron* 46:37–49.
- Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A (2005) Light Activation of Channelrhodopsin-2 in Excitable Cells of *Caenorhabditis elegans* Triggers Rapid Behavioral Responses. *Current Biology* 15:2279–2284.
- Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, Bamberg E (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc Natl Acad Sci USA* 100:13940–13945.
- Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA* 90:8424–8428.
- Naka-Kaneda H, Nakamura S, Igarashi M, Aoi H, Kanki H, Tsuyama J, Tsutsumi S, Aburatani H, Shimazaki T, Okano H (2014) The miR-17/106-p38 axis is a key regulator of the neurogenic-to-gliogenic transition in developing neural stem/progenitor cells. *Proc Natl Acad Sci USA* 111:1604–1609.
- Nakayama T, Momoki-Soga T, Yamaguchi K, Inoue N (2004) Efficient production of neural stem cells and neurons from embryonic stem cells. *Neuroreport* 15:487–491.
- Navarro X, Vivó M, Valero-Cabré A (2007) Neural plasticity after peripheral nerve injury and regeneration. *Prog Neurobiol* 82:163–201.

- Nistor G, Siegenthaler MM, Poirier SN, Rossi S, Poole AJ, Charlton ME, McNeish JD, Airriess CN, Keirstead HS (2011) Derivation of high purity neuronal progenitors from human embryonic stem cells. Cooney AJ, ed. PLoS ONE 6:e20692.
- Noble J, Munro CA, Prasad VSSV, Midha R (1998) Analysis of Upper and Lower Extremity Peripheral Nerve Injuries in a Population of Patients with Multiple Injuries. *The Journal of Trauma: Injury, Infection, and Critical Care* 45:116–122.
- Noonan VK, Fingas M, Farry A, Baxter D, Singh A, Fehlings MG, Dvorak MF (2012) Incidence and Prevalence of Spinal Cord Injury in Canada: A National Perspective. *Neuroepidemiology* 38:219–226.
- Nógrádi A, Pajer K, Márton G (2011) The role of embryonic motoneuron transplants to restore the lost motor function of the injured spinal cord. *Ann Anat* 193:362–370.
- Nógrádi A, Szabó A (2008) Transplantation of embryonic neurones to replace missing spinal motoneurons. *Restor Neurol Neurosci* 26:215–223.
- Nógrádi A, Vrbová G (1996) Improved motor function of denervated rat hindlimb muscles induced by embryonic spinal cord grafts. *Eur J Neurosci* 8:2198–2203.
- O'Connell K, Whitesell JD, al E (2008) Localization and mobility of the delayed-rectifier K⁺ channel Kv2. 1 in adult cardiomyocytes. *American Journal of ...*
- Oberlin C, Béal D, Leechavengvongs S, Salon A, Dauge MC, Sarcy JJ (1994) Nerve transfer to biceps muscle using a part of ulnar nerve for C5–C6 avulsion of the brachial plexus: Anatomical study and report of four cases. *J Hand Surg Am* 19:232–237.
- Odorico JS, Kaufman DS, Thomson JA (2001) Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* 19:193–204.
- Ollivier-Lanvin K, Lemay MA, Tessler A, Burns AS (2009) Neuromuscular transmission failure and muscle fatigue in ankle muscles of the adult rat after spinal cord injury. *Journal of Applied Physiology* 107:1190–1194.
- Park C-H, Minn Y-K, Lee J-Y, Choi DH, Chang M-Y, Shim J-W, Ko J-Y, Koh H-C, Kang MJ, Kang JS, Rhie D-J, Lee Y-S, Son H, Moon SY, Kim K-S, Lee S-H (2005) In vitro and in vivo analyses of human embryonic stem cell-derived dopamine neurons. *J Neurochem* 92:1265–1276.
- Park I-H, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ (2008) Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451:141–146.
- Patterson M, Gaeta X, Loo K, Edwards M, Smale S, Cinkornpumin J, Xie Y, Listgarten J, Azghadi S, Douglass SM, Pellegrini M, Lowry WE (2014) let-7 miRNAs can act through notch to regulate human gliogenesis. *Stem Cell Reports* 3:758–773.
- Patterson MF, Stephenson GMM, Stephenson DG (2006) Denervation produces different single fiber phenotypes in fast- and slow-twitch hindlimb muscles of the rat. *Am J Physiol, Cell Physiol* 291:C518–C528.

- Peljto M, Dasen JS, Mazzoni EO, Jessell TM, Wichterle H (2010) Functional diversity of ESC-derived motor neuron subtypes revealed through intraspinal transplantation. *Cell Stem Cell* 7:355–366.
- Peljto M, Wichterle H (2011) Programming embryonic stem cells to neuronal subtypes. *Curr Opin Neurobiol* 21:43–51.
- Perry VH, Brown MC (1992) Macrophages and nerve regeneration. *Curr Opin Neurobiol* 2:679–682.
- Pfeifer K, Vroemen M, Blesch A, Weidner N (2004) Adult neural progenitor cells provide a permissive guiding substrate for corticospinal axon growth following spinal cord injury. *Eur J Neurosci* 20:1695–1704.
- Pirouzmand F (2010) Epidemiological trends of spine and spinal cord injuries in the largest Canadian adult trauma center from 1986 to 2006. *J Neurosurg Spine* 12:131–140.
- Plenk H (2011) The role of materials biocompatibility for functional electrical stimulation applications. *Artificial organs* 35:237–241.
- Popovic D, Gordon T, Rafuse VF, Prochazka A (1991) Properties of implanted electrodes for functional electrical stimulation. *Ann Biomed Eng* 19:303–316.
- Popović DB (2014) Advances in functional electrical stimulation (FES). *Journal of Electromyography and Kinesiology* 24:795–802.
- Prochazka A, Mushahwar VK, McCreery DB (2001) Neural prostheses. *J Physiol (Lond)* 533:99–109.
- Qin W, Bauman WA, Cardozo C (2010) Bone and muscle loss after spinal cord injury: organ interactions. *Ann N Y Acad Sci* 1211:66–84.
- Quinlan GA, Williams EA, Tan SS, Tam PP (1995) Neuroectodermal fate of epiblast cells in the distal region of the mouse egg cylinder: implication for body plan organization during early embryogenesis. *Development* 121:87–98.
- Quy PN, Kuma A, Pierre P, Mizushima N (2013) Proteasome-dependent Activation of Mammalian Target of Rapamycin Complex 1 (mTORC1) Is Essential for Autophagy Suppression and Muscle Remodeling Following Denervation. *J Biol Chem* 288:1125–1134.
- Rafuse VF, Gordon T, Orozco R (1992) Proportional enlargement of motor units after partial denervation of cat triceps surae muscles. *J Neurophysiol* 68:1261–1276.
- Rafuse VF, Soundararajan P, Leopold C, Robertson HA (2005) Neuroprotective properties of cultured neural progenitor cells are associated with the production of sonic hedgehog. *NSC* 131:899–916.
- Ragnarsson KT (2008) Functional electrical stimulation after spinal cord injury: current use, therapeutic effects and future directions. *Spinal Cord* 46:255–274.
- Ramer LM, Ramer MS, Steeves JD (2005) Setting the stage for functional repair of spinal cord injuries: a cast of thousands. *Spinal Cord* 43:134–161.

Rassendren FA, Bloch-Gallego E, Tanaka H, Henderson CE (1992) Levels of mRNA coding for motoneuron growth-promoting factors are increased in denervated muscle. *Proc Natl Acad Sci USA* 89:7194–7198.

Reier PJ (2004) Cellular transplantation strategies for spinal cord injury and translational neurobiology. *NeuroRx* 1:424–451.

Renton AE et al. (2011) A Hexanucleotide Repeat Expansion in C9ORF72 Is the Cause of Chromosome 9p21-Linked ALS-FTD. *Neuron* 72:257–268.

Reuter I, Tai YF, Pavese N, Chaudhuri KR, Mason S, Polkey CE, Clough C, Brooks DJ, Barker RA, Piccini P (2008) Long-term clinical and positron emission tomography outcome of fetal striatal transplantation in Huntington's disease. *J Neurol Neurosurg Psychiatr* 79:948–951.

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255:1707–1710.

Reynolds ML, Woolf CJ (1992) Terminal Schwann cells elaborate extensive processes following denervation of the motor endplate. *J Neurocytol* 21:50–66.

Richardson PM, Issa VMK (1984) Peripheral injury enhances central regeneration of primary sensory neurones. , Published online: 28 June 1984; | doi:101038/309791a0 309:791–793.

Ritter A, Wenner P, Ho S, Whelan PJ, O'donovan MJ (1999) Activity patterns and synaptic organization of ventrally located interneurons in the embryonic chick spinal cord. *J Neurosci* 19:3457–3471.

Robinson LR (2000) Traumatic injury to peripheral nerves. *Muscle Nerve* 23:863–873.

Rosato-Siri MD, Zoccolan D, Furlan F, Ballerini L (2004) Interneurone bursts are spontaneously associated with muscle contractions only during early phases of mouse spinal network development: a study in organotypic cultures. *Eur J Neurosci* 20:2697–2710.

Rosenberg LJ, Zai LJ, Wrathall JR (2005) Chronic alterations in the cellular composition of spinal cord white matter following contusion injury. *Glia* 49:107–120.

Rossant J, Chazaud C, Yamanaka Y (2003) Lineage allocation and asymmetries in the early mouse embryo. *Philos Trans R Soc Lond, B, Biol Sci* 358:1341–8–discussion1349.

Ruijs ACJ, Jaquet J-B, Kalmijn S, Giele H, Hovius SER (2005) Median and ulnar nerve injuries: a meta-analysis of predictors of motor and sensory recovery after modern microsurgical nerve repair. *Plast Reconstr Surg* 116:484–94–discussion495–6.

Rutishauser U, Landmesser L (1991) Polysialic acid on the surface of axons regulates patterns of normal and activity-dependent innervation. *Trends Neurosci* 14:528–532.

Rüschenschmidt C, Koch PG, Brüstle O, Beck H (2005) Functional properties of ES cell-derived neurons engrafted into the hippocampus of adult normal and chronically epileptic rats. *Epilepsia* 46 Suppl 5:174–183.

Sabelström H, Stenudd M, Frisén J (2014) Neural stem cells in the adult spinal cord. *Exp Neurol* 260:44–49.

Sabelström H, Stenudd M, Réu P, Dias DO, Elfineh M, Zdunek S, Damberg P, Göritz C, Frisé J (2013) Resident neural stem cells restrict tissue damage and neuronal loss after spinal cord injury in mice. *Science* 342:637–640.

Saito H, Dahlin LB (2008) Expression of ATF3 and axonal outgrowth are impaired after delayed nerve repair. *BMC Neurosci* 9:88.

Sakuma M, Gorski G, Sheu S-H, Lee S, Barrett LB, Singh B, Omura T, Latremoliere A, Woolf CJ (2015) Lack of motor recovery after prolonged denervation of the neuromuscular junction is not due to regenerative failure Gaspar P, ed. *Eur J Neurosci*:n/a–n/a.

Salmons S, Ashley Z, Sutherland H, Russold MF, Li F, Jarvis JC (2005) Functional Electrical Stimulation of Denervated Muscles: Basic Issues. *Artificial organs* 29:199–202.

Scain A-L, Le Corronc H, Allain A-E, Muller E, Rigo J-M, Meyrand P, Branchereau P, Legendre P (2010) Glycine release from radial cells modulates the spontaneous activity and its propagation during early spinal cord development. *Journal of Neuroscience* 30:390–403.

Scheib J, Hoke A (2013) Advances in peripheral nerve regeneration. *Nat Rev Neurol* 9:668–676.

Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M (2013) Mechanisms regulating skeletal muscle growth and atrophy. *FEBS Journal* 280:4294–4314.

Seddon H (1975) *Surgical Disorders of the Peripheral Nerves*. Churchill Livingstone.

Sekhon LH, Fehlings MG (2001) Epidemiology, demographics, and pathophysiology of acute spinal cord injury. *Spine* 26:S2–S12.

Selecki BR, Ring IT, Simpson DA, Vanderfield GK, Sewell MF (1982) TRAUMA TO THE CENTRAL AND PERIPHERAL NERVOUS SYSTEMS PART II: A STATISTICAL PROFILE OF SURGICAL TREATMENT NEW SOUTH WALES 1977. *ANZ J Surg* 52:111–116.

Shields RK, Dudley-Javoroski S (2007) Musculoskeletal adaptations in chronic spinal cord injury: effects of long-term soleus electrical stimulation training. *Neurorehabilitation and neural repair* 21:169–179.

Shih C-C, Forman SJ, Chu P, Slovak M (2007) Human embryonic stem cells are prone to generate primitive, undifferentiated tumors in engrafted human fetal tissues in severe combined immunodeficient mice. *Stem Cells Dev* 16:893–902.

Sieradzan K, Vrbová G (1989) Replacement of missing motoneurons by embryonic grafts in the rat spinal cord. *NSC* 31:115–130.

Sieradzan K, Vrbová G (1991) Factors influencing survival of transplanted embryonic motoneurons in the spinal cord of adult rats. *Exp Neurol* 114:286–299.

Siironen J, Sandberg M, Vuorinen V, Røyttä M (1992) Expression of type I and III collagens and fibronectin after transection of rat sciatic nerve. Reinnervation compared with denervation. *Lab Invest* 67:80–87.

- Singh A, Tetreault L, Kalsi-Ryan S, Nouri A, Fehlings MG (2014) Global prevalence and incidence of traumatic spinal cord injury. *Clin Epidemiol* 6:309–331.
- Smith AJ, Nelson NG, Oommen S, Hartjes KA, Folmes CD, Terzic A, Nelson TJ (2012) Apoptotic susceptibility to DNA damage of pluripotent stem cells facilitates pharmacologic purging of teratoma risk. *Stem Cells Transl Med* 1:709–718.
- Son EY, Ichida JK, Wainger BJ, Toma JS, Rafuse VF, Woolf CJ, Eggan K (2011) Conversion of Mouse and Human Fibroblasts into Functional Spinal Motor Neurons. *Stem Cell* 9:205–218.
- Son YJ, Thompson WJ (1995) Nerve sprouting in muscle is induced and guided by processes extended by Schwann cells. *Neuron* 14:133–141.
- Sortwell CE, Pitzer MR, Collier TJ (2000) Time Course of Apoptotic Cell Death within Mesencephalic Cell Suspension Grafts: Implications for Improving Grafted Dopamine Neuron Survival. *Exp Neurol* 165:268–277.
- Soundararajan P, Fawcett JP, Rafuse VF (2010) Guidance of postural motoneurons requires MAPK/ERK signaling downstream of fibroblast growth factor receptor 1. *Journal of Neuroscience* 30:6595–6606.
- Soundararajan P, Lindsey BW, Leopold C, Rafuse VF (2007) Easy and rapid differentiation of embryonic stem cells into functional motoneurons using sonic hedgehog-producing cells. *Stem Cells* 25:1697–1706.
- Soundararajan P, Miles GB, Rubin LL, Brownstone RM, Rafuse VF (2006) Motoneurons derived from embryonic stem cells express transcription factors and develop phenotypes characteristic of medial motor column neurons. *Journal of Neuroscience* 26:3256–3268.
- Spinner RJ, Kline DG (2000) Surgery for peripheral nerve and brachial plexus injuries or other nerve lesions. *Muscle Nerve* 23:680–695.
- Squecco R, Carraro U, Kern H, Pond A, Adami N, Biral D, Vindigni V, Boncompagni S, Pietrangelo T, Bosco G, Fanò G, Marini M, Abruzzo PM, Germinario E, Danieli-Betto D, Protasi F, Francini F, Zampieri S (2009) A subpopulation of rat muscle fibers maintains an assessable excitation-contraction coupling mechanism after long-standing denervation despite lost contractility. *J Neuropathol Exp Neurol* 68:1256–1268.
- Statland JM, Barohn RJ, McVey AL, Katz JS, Dimachkie MM (2015) Patterns of Weakness, Classification of Motor Neuron Disease, and Clinical Diagnosis of Sporadic Amyotrophic Lateral Sclerosis. *Neurologic clinics* 33:735–748.
- Stein V, Hermans-Borgmeyer I, Jentsch TJ, Hübner CA (2004) Expression of the KCl cotransporter KCC2 parallels neuronal maturation and the emergence of low intracellular chloride. *J Comp Neurol* 468:57–64.
- Steinbeck JA, Studer L (2015) Moving Stem Cells to the Clinic: Potential and Limitations for Brain Repair. *Neuron* 86:187–206.
- Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SWM,

- Barres BA (2007) The classical complement cascade mediates CNS synapse elimination. *Cell* 131:1164–1178.
- Straight AF, Cheung A, Limouze J, Chen I, Westwood NJ, Sellers JR, Mitchison TJ (2003) Dissecting Temporal and Spatial Control of Cytokinesis with a Myosin II Inhibitor. *Science* 299:1743–1747.
- Streit J, Tschertter A, Heuschkel MO, Renaud P (2001) The generation of rhythmic activity in dissociated cultures of rat spinal cord. *Eur J Neurosci* 14:191–202.
- Strübing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM (1995) Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. *Mech Dev* 53:275–287.
- Sulaiman OA, Gordon T (2000) Effects of short- and long-term Schwann cell denervation on peripheral nerve regeneration, myelination, and size. *Glia* 32:234–246.
- Sulaiman OAR, Gordon T (2009) Role of chronic Schwann cell denervation in poor functional recovery after nerve injuries and experimental strategies to combat it. *65:105–114*.
- Sunderland S (1978a) *Nerves And Nerve Injuries*. Churchill Livingstone.
- Sunderland S (1978b) *Sunderland: Nerves and injuries* - Google Scholar. Edinburgh: Churchill & Livingstone.
- Sürmeli G, Akay T, Ippolito GC, Tucker PW, Jessell TM (2011) Patterns of spinal sensory-motor connectivity prescribed by a dorsoventral positional template. *Cell* 147:653–665.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872.
- Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
- Tam PP, Zhou SX (1996) The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. *Dev Biol* 178:124–132.
- Tang C, Lee AS, Volkmer J-P, Sahoo D, Nag D, Mosley AR, Inlay MA, Ardehali R, Chavez SL, Pera RR, Behr B, Wu JC, Weissman IL, Drukker M (2011) An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. *Nat Biotechnol* 29:829–834.
- Tator CH, Fehlings MG (1991) Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. *J Neurosurg* 75:15–26.
- Terenghi G (1999) Peripheral nerve regeneration and neurotrophic factors. *J Anat* 194 (Pt 1):1–14.
- Tetzlaff W, Okon EB, Karimi-Abdolrezaee S, Hill CE, Sparling JS, Plemel JR, Plunet WT, Tsai EC, Baptiste D, Smithson LJ, Kawaja MD, Fehlings MG, Kwon BK (2011) A systematic review of cellular transplantation therapies for spinal cord injury. *J Neurotrauma* 28:1611–1682.

- Thaler J, Harrison K, Sharma K, Lettieri K, Kehrl J, Pfaff SL (1999) Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. *Neuron* 23:675–687.
- Theele DP, Schrimsher GW, Reier PJ (1996) Comparison of the growth and fate of fetal spinal iso- and allografts in the adult rat injured spinal cord. *Exp Neurol* 142:128–143.
- Thomas CK, Erb DE, Grumbles RM, Bunge RP (2000) Embryonic cord transplants in peripheral nerve restore skeletal muscle function. *J Neurophysiol* 84:591–595.
- Thomas CK, Sesodia S, Erb DE, Grumbles RM (2003) Properties of medial gastrocnemius motor units and muscle fibers reinnervated by embryonic ventral spinal cord cells. *Exp Neurol* 180:25–31.
- Thomas CK, Zaidner EY, Calancie B, Broton JG, Bigland-Ritchie BR (1997) Muscle Weakness, Paralysis, and Atrophy after Human Cervical Spinal Cord Injury. *Exp Neurol* 148:414–423.
- Thomas CK, Zijdewind I (2006) Fatigue of muscles weakened by death of motoneurons. *Muscle Nerve* 33:21–41.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147.
- Toma JS, Shettar BC, Chipman PH, Pinto DM, Borowska JP, Ichida JK, Fawcett JP, Zhang Y, Eggan K, Rafuse VF (2015) Motoneurons derived from induced pluripotent stem cells develop mature phenotypes typical of endogenous spinal motoneurons. *Journal of Neuroscience* 35:1291–1306.
- Tötösy de Zepetnek JE, Zung HV, Erdebil S, Gordon T (1992) Innervation ratio is an important determinant of force in normal and reinnervated rat tibialis anterior muscles. *J Neurophysiol* 67:1385–1403.
- Traxinger K, Kelly C, Johnson BA, Lyles RH, Glass JD (2013) Prognosis and epidemiology of amyotrophic lateral sclerosis: Analysis of a clinic population, 1997-2011. *Neurol Clin Pract* 3:313–320.
- Tsang TE, Khoo PL, Jamieson RV, Zhou SX, Ang SL, Behringer R, Tam PP (2001) The allocation and differentiation of mouse primordial germ cells. *Int J Dev Biol* 45:549–555.
- Ung R-V, Rouleau P, Guertin PA (2010) Effects of co-administration of clenbuterol and testosterone propionate on skeletal muscle in paraplegic mice. *J Neurotrauma* 27:1129–1142.
- van Bremen T, Vogt CC, Send T, Fleischmann BK, Bruegmann T, Sasse P (2015) Optogenetic control of contractile function in skeletal muscle. *Nat Comms* 6:1–8.
- Van Pelt J, Corner MA, Wolters PS, Rutten WLC, Ramakers GJA (2004) Longterm stability and developmental changes in spontaneous network burst firing patterns in dissociated rat cerebral cortex cell cultures on multielectrode arrays. *Neurosci Lett* 361:86–89.
- Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Südhof TC, Wernig M (2010) Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463:1035–1041.

- Vrieseling E, Arber S (2006) Target-induced transcriptional control of dendritic patterning and connectivity in motor neurons by the ETS gene *Pea3*. *Cell* 127:1439–1452.
- Wagner M, Han B, Jessell TM (1992) Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. *Development* 116:55–66.
- Wang H, Sugiyama Y, Hikima T, Sugano E, Tomita H, Takahashi T, Ishizuka T, Yawo H (2009) Molecular determinants differentiating photocurrent properties of two channelrhodopsins from *Chlamydomonas*. *J Biol Chem* 284:5685–5696.
- Wang L, Klein R, Zheng B, Marquardt T (2011) Anatomical Coupling of Sensory and Motor Nerve Trajectory via Axon Tracking. *Neuron* 71:263–277.
- Wang XF, Cynader MS (1999) Effects of astrocytes on neuronal attachment and survival shown in a serum-free co-culture system. *Brain Res Brain Res Protoc* 4:209–216.
- Weerakkody TN, Patel TP, Yue C, Takano H, Anderson HC, Meaney DF, Coulter DA, Wolfe JH (2013) Engraftment of nonintegrating neural stem cells differentially perturbs cortical activity in a dose-dependent manner. *Mol Ther* 21:2258–2267.
- Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA (1996) Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci* 16:7599–7609.
- Wen L, Wang H, Tanimoto S, Egawa R, Matsuzaka Y, Mushiake H, Ishizuka T, Yawo H (2010) Opto-current-clamp actuation of cortical neurons using a strategically designed channelrhodopsin. *Brezina V, ed. PLoS ONE* 5:e12893.
- Wenner P, O'donovan MJ (2001) Mechanisms that initiate spontaneous network activity in the developing chick spinal cord. *J Neurophysiol* 86:1481–1498.
- Wernig M, Benninger F, Schmandt T, Rade M, Tucker KL, Büssow H, Beck H, Brüstle O (2004) Functional integration of embryonic stem cell-derived neurons in vivo. *Journal of Neuroscience* 24:5258–5268.
- Westmoreland JJ, Hancock CR, Condie BG (2001) Neuronal development of embryonic stem cells: a model of GABAergic neuron differentiation. *Biochemical and Biophysical Research Communications* 284:674–680.
- White TE, Lane MA, Sandhu MS, O'Steen BE, Fuller DD, Reier PJ (2010) Neuronal progenitor transplantation and respiratory outcomes following upper cervical spinal cord injury in adult rats. *Exp Neurol* 225:231–236.
- Wichterle H, Lieberam I, Porter JA, Jessell TM (2002) Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110:385–397.
- Willand MP, Lopez JP, de Bruin H, Fahnstock M, Holmes M, Bain JR (2011) A New System and Paradigm for Chronic Stimulation of Denervated Rat Muscle. *J Med Biol Eng* 31:87–92.
- Wilson SI, Edlund T (2001) Neural induction: toward a unifying mechanism. *Nat Neurosci* 4 Suppl:1161–1168.

- Wong FSY, Chan BP, Lo ACY (2014) Carriers in cell-based therapies for neurological disorders. *Int J Mol Sci* 15:10669–10723.
- Wu WL, Ziskind-Conhaim L, Sweet MA (1992) Early development of glycine- and GABA-mediated synapses in rat spinal cord. *J Neurosci* 12:3935–3945.
- Wu Y, Collier L, Pan J, Qin W, Bauman WA, Cardozo CP (2012) Testosterone reduced methylprednisolone-induced muscle atrophy in spinal cord-injured rats. *Spinal Cord* 50:57–62.
- Wyatt LA, Keirstead HS (2012) Stem cell-based treatments for spinal cord injury. *Prog Brain Res* 201:233–252.
- Xie H, Ziskind-Conhaim L (1995) Blocking Ca²⁺-dependent synaptic release delays motoneuron differentiation in the rat spinal cord. *J Neurosci* 15:5900–5911.
- Yang JF, Stein RB, Jhamandas J, Gordon T (1990) Motor unit numbers and contractile properties after spinal cord injury. *Ann Neurol* 28:496–502.
- Yang LJ-S, Chang KW-C, Chung KC (2012) A systematic review of nerve transfer and nerve repair for the treatment of adult upper brachial plexus injury. *Neurosurgery* 71:417–29–discussion429.
- Yohn DC, Miles GB, Rafuse VF, Brownstone RM (2008) Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy. *Journal of Neuroscience* 28:12409–12418.
- You S, Petrov T, Chung PH, Gordon T (1997) The expression of the low affinity nerve growth factor receptor in long-term denervated Schwann cells. *Glia* 20:87–100.
- Zeman RJ, Zhao J, Zhang Y, Zhao W, Wen X, Wu Y, Pan J, Bauman WA, Cardozo C (2009) Differential skeletal muscle gene expression after upper or lower motor neuron transection. *Pflugers Arch - Eur J Physiol* 458:525–535.
- Zeng X, Rao MS (2007) Human embryonic stem cells: long term stability, absence of senescence and a potential cell source for neural replacement. *NSC* 145:1348–1358.
- Zhang F, Fischer KA (2002) End-to-side neurorrhaphy. *Microsurgery* 22:122–127.
- Zhang P, Chen X, Fan M (2007) Signaling mechanisms involved in disuse muscle atrophy. *Med Hypotheses* 69:310–321.
- Zhang Y, Narayan S, Geiman E, Lanuza G, Velasquez T, Shanks B, Akay T, Dyck J, Pearson K, Gosgnach S (2008) V3 Spinal Neurons Establish a Robust and Balanced Locomotor Rhythm during Walking. *Neuron* 60:84–96.
- Zhao C, Veltri K, Li S, Bain JR, Fahnstock M (2004) NGF, BDNF, NT-3, and GDNF mRNA expression in rat skeletal muscle following denervation and sensory protection. *J Neurotrauma* 21:1468–1478.
- Zwaka TP, Thomson JA (2005) A germ cell origin of embryonic stem cells? *Development* 132:227–233.