Self-tracing Human Induced Pluripotent Stem Cell-Derived Neural Stem Cells to Map Transplant Integration

by

Priscilla Chan

A thesis submitted in conformity with the requirements
for the degree of Master of Science

Institute of Medical Science
University of Toronto

Self-tracing Human Induced Pluripotent Stem Cell-Derived Neural Stem Cells to Map Transplant Integration

Priscilla Chan

Master of Science

Institute of Medical Science
University of Toronto

2019

Abstract

Spinal cord injury (SCI) is a devastating condition that leaves patients with lifelong sensory, motor, and autonomic deficits. Stem cell therapies offer an attractive approach to repairing and regenerating the injured spinal cord. While previous studies have shown that human neural stem cells (NSCs) can improve locomotion, few have been able to demonstrate graft-host integration, in part, due to ineffective viral and non-viral tracing methods. To overcome this challenge, we engineered NSCs to express both antero- and retrograde trans-synaptic tracers to map transplant integration. The resultant self-tracing NSCs retained typical stem cell properties as control NSCs and differentiated into functional neurons. Furthermore, they successfully traced primary rat cortical neurons *in vitro*. Preliminary *in vivo* data suggest self-tracing NSCs may also trace host neurons. This exciting proof-of-concept shows promise as a tool to delineate synaptically integrated sensorimotor pathways involved in stem cell-mediated recovery.

Acknowledgments

Having joined the Fehlings Lab as an undergraduate research student, I chose to stay for my master's degree not only for the science, but also for the people. After all, no graduate student could survive without the help of their supervisor, colleagues, and friends.

To Dr. Michael Fehlings: Thank you for your constant support not only during my graduate studies, but also since my undergraduate years. You have provided me with a unique learning environment where I have grown both personally and professionally.

To Drs. Michael Laflamme and Charles Tator: Thank you for providing me with invaluable feedback and advice throughout my project.

To my lab family, past and present: Thank you for making the Fehlings Lab such a wonderful work environment. We've shared many memories which I hope to never forget.

To Dr. Mahmood Chamankhah: Thank you for seeing potential in me from the very beginning. You taught me how to stay curious and maintain a healthy dose of skepticism. Without your patience and guidance, I would not be where I am today. I am forever indebted.

To Drs. Chris Ahuja, Mamad Khazaei, and Sasha Velumian: Thank you for showing me that creativity has a place in science. Your excitement for research is contagious.

To Dr. Anna Badner: Thank you for showing me that there is a light at the end of the tunnel. You are never afraid to remind me when I should get my act together or give myself a break. Who knew sharing some cookies would lead to a lifetime of friendship?

Above all, to my family: Thank you for your endless love. You are always there to drop off food or pick me up after a long day at the lab. You are always willing to listen to my stories about my cells and rats. You are always understanding when I work weekends for a squeeze shift. Most importantly, you are always there to pick me up when I fall so that I never let me lose sight of my dreams.

Statement of Contributions

Chapter 2

Dr. Christopher Ahuja assisted with optimizing FACS in section 2.1.3.

Dr. Mohammad Khazaei assisted with developing differentiation protocols in sections 2.3.

Dr. Alexander Velumian assisted with patch clamp protocols in section 2.3.4.

Dr. Michael Lane and Margo Randelman assisted with developing and troubleshooting the pseudorabies virus tracing protocol in section 2.5.4.

Chapter 3

Drs. Christopher Ahuja and Mohammad Khazaei assisted with initial experimental design and optimization of plasmid subcloning in sections 3.1.1.

Dr. Mohammad Khazaei provided hiPSC-NSCs.

Dr. Alexander Velumian assisted with data collection in section 3.3.3.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

AANS	American Association of Neurological Surgeons
AAV	Adeno-associated virus
aCSF	Artificial cerebrospinal fluid
AIS	ASIA Impairment Scale
ANOVA	Analysis of variance
APC	Allophycocyanin
AQP4	Aquaporin 4
ASIA	American Spinal Injury Association
BBB	Basso Beattie Bresnahan locomotor rating scale
BBS	Berg Balance Scale
BDA	Biotinylated dextran amine
BDNF	Brain-derived neurotrophic factor
BMSC	Bone marrow stem cell
BSCB	Blood-spinal cord barrier
cAMP	Cyclic adenosine monophosphate
CNS	Congress of Neurological Surgeons
CRISPR	Clustered regularly interspaced short palindromic repeats
CST	Corticospinal tract
DAB	Diaminobenzidine
DCML	Dorsal column medial lemniscus
DLG4	Discs large MAGUK scaffold protein 4
DNA	Deoxyribonucleic acid
EF1a	Elongation factor 1 alpha
EGF	Epithelial growth factor
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDP	Flexor digitorum profundus
FGF2	Fibroblast growth factor 2
GAP43	Growth-associated protein 43
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HBSS	Hank's Balanced Salt Solution
HGF	Hepatocyte growth factor
hH11	Human Hipp11
HRP	Horseradish peroxidase
HuCNS-SC	Human Central Nervous System Stem Cells
IBB	Irvine Basso Beattie forelimb recovery scale
IGF	Insulin-like growth factor
iPSC	Induced pluripotent stem cell
IR	Infrared
IRES	Internal ribosomal entry site
ISNCSCI	International Standards for Neurological Classification of Spinal Cord Injury
ISSCR	International Society for Stem Cell Research
ITR	Inverted terminal repeat
HSV	Herpes simplex virus
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IRES	Internal ribosomal entry site
LY	Lucifer yellow
MAP2	Microtubule-associated protein 2
MPSS	Methylprednisolone sodium succinate
MSC	Mesenchymal stem cell
MSI1	Musashi 1
NASCIS	National acute spinal cord injury study
NES	Nestin
NIM	Neural induction media
NMM	Neural maintenance media
NSC	Neural stem cell
NT3	Neurotrophin 3
PAX6	Paired box protein 6

pB	piggyBac
PBS	Phosphate buffered saline
PDGFAA	Platelet-derived growth factor AA
PFA	Paraformaldehyde
PLO	Poly-L-ornithine
PRV	Pseudorabies virus
RISCIS	Riluzole in spinal cord injury study
RNA	Ribonucleic acid
RST	Rubrospinal tract
RT-PCR	Real-time polymerase chain reaction
SCI	Spinal cord injury
SCIM	Spinal cord independence measure
SCT	Spinocerebellar tract
SEM	Standard error of mean
SFM	Serum free media
Shh	Sonic hedgehog
SOX1	SRY-box 1
SOX2	SRY-box 2
STASCIS	Surgical timing in acute spinal cord injury study
STT	Spinothalamic tract
T3	Triiodothyronine
TBI	Traumatic brain injury
Tet-On	Tetracycline-on
TeTx	Tetanus toxin
TTC	Tetanus toxin fragment C
TUBB3	Beta-3 tubulin
UCOE	Ubiquitous chromatic opening element
VIM	Vimentin
VST	Vestibulospinal tract
WGA	Wheat germ agglutinin
WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element

Chapter 1
Literature Review

1 Literature Review

1.1 Spinal cord injury (SCI) overview

In North American alone, traumatic spinal cord injury (SCI) leaves more than 1 million patients with profound and long-term sensory, motor, and autonomic deficits. While the sensorimotor disabilities are commonly discussed, patients may also experience respiratory depression, autonomic dysreflexia, increased risk of thromboembolism, pressure ulcers, and bowel dysfunction depending on the level and severity of injury (Tator, 2006). Taken together, patients experience a dramatic difference in their quality of life.

Adding to the personal costs of SCI are significant societal costs associated with expensive health care treatment, rehabilitation, and loss of productivity (Munce et al., 2013). It is estimated that the lifetime economic burden of SCI ranges from 1.5 million to 3 million CAD per patient (Krueger et al., 2013). The staggering costs to treat SCI has driven researchers to develop novel therapies that produce major functional improvements. To date, the field of SCI has led a healthy number of clinical trials despite being a relatively small field, with only 11 000 new cases annually in North America. Unfortunately, these trials have largely been unsuccessful, and a lack of an effective treatment remains.

1.1.1 Epidemiology and etiology

The incidence of SCI is approximately 30 to 40 cases per million individuals in Canada (Singh et al., 2014) and the United States ("Spinal Cord Injury Facts and Figures at a Glance," 2014; "Spinal Cord Injury Facts and Figures at a Glance," 2012). This is considerably higher than figures reported in other developed regions of the world, including Western Europe and Australia, which are closer to 15 cases per million (Chen et al., 2013). Globally, SCI prevalence has been estimated between 236 to 1173 per million (Cripps et al., 2011). This is likely a gross underestimate given that many largely populated countries are missing a national SCI database.

Despite differences in the number of SCI patients worldwide, it appears that motor vehicle accidents are a common major cause, accounting for approximately 50% of all cases (Cripps et

al., 2011). This is followed by a high rate of slip-and-falls, especially in developed regions with aged populations. In contrast, SCI due to violence is more common in developing countries. The remaining cases of SCI can be attributed to either sports/recreational injuries or workplace injuries. It is also important to note that many patients also experience multisystem trauma, further complicating a patient's recovery (Burney et al., 1993; Krause et al., 2010).

Typically, males are three to four times more likely to incur an SCI compare to females and the average age of SCI patients is 35 years old. In fact, young males aged 20 to 30 comprise the majority of SCI patients (Sekhon and Fehlings, 2001). However, more recent data suggests that an increasing number of older individuals are experiencing SCI after falling (Jain et al., 2015; Wilson et al., 2014). Mirroring the ageing population, this the proportion of elderly patients is only expected to grow. Unfortunately, older individuals are also less likely to recover from injury.

Given the various etiologies of SCI, the patient population is highly heterogeneous, both in terms injury level and severity. 55 to 75% of all SCIs occur at the cervical level with a relatively even distribution of injuries occurring at the thoracic and lumbar regions (Pickett et al., 2006; Sekhon and Fehlings, 2001). Once injured, impairments occur at and below the level of injury. From the cervical level C5 to thoracic T1, the main muscle groups affected at the elbow flexors, wrist flexors, elbow extensors, finger flexors, and finger abductors, respectively (Ditunno et al., 1994; Kirshblum et al., 2011). Meanwhile, the lumbar level L2 to sacral S1 innervates the hip flexors, knee extensors, ankle dorsiflexors, long toe extensors, and ankle plantar flexors. As a result, cervical SCI patients typically suffer from greater impairments. Moreover, cervical SCI often interrupts descending bulbospinal respiratory pathways leading to increased respiratory complications (Zimmer et al., 2007).

Clinicians have also standardized the classification of neurologic deficits. By assessing the presence or absence of key muscle functions and sensory perceptions, they are able to determine the severity of SCI (Table 1). It is rare for patients to present with a complete SCI, defined by the American Spinal Injury Association (ASIA) Impairment Scale (AIS, or International Standards for Neurological Classification of Spinal Cord Injury, ISNCSCI) as having no motor or sensory function at all. However, of the patients deemed AIS A, fewer than 5% convert to AIS B, C, or D 5 years post-injury (Kirshblum et al., 2011).

1.1.2 Mortality rate

Although the survival rates of SCI patients have greatly improved over time, their mortality rates continue to exceed those of age-matched controls. An estimated 4% to 17% of SCI patients do not survive beyond initial hospitalization. Patients who are discharged have annual mortality rates of 3.8%, 1.6% and 1.2% in the first, second, and subsequent years after injury (Ahuja et al., 2017). Previously, urinary tract infections were thought to be the leading cause of death in these chronic patients, but later studies suggest that respiratory complications are to blame (De Vivo et al., 1989). The presence of multisystem trauma, older age, higher level, and more severe injuries further increase the risk of mortality and decrease the expected lifespan of an SCI patient.

1.1.3 Pathophysiology

The sensorimotor deficits caused by spinal cord injury results from the mechanical damage to the spinal cord. This initial insult has been termed primary injury and encompasses a variety of mechanisms, including contusion, compression, shear, stretch, and transection. Aside from injury prevention, there is little to nothing that can be done to reverse the primary injury. Instead, researchers place a greater emphasis on the secondary injury phase of SCI, a series of cellular and molecular events that propagate tissue damage. Although secondary injury is not entirely harmful, there is a well-defined timeline of mechanisms that can be targeted by potential therapeutic interventions to ameliorate recovery.

Table 1 American Spinal Injury Association (ASIA) Impairment Scale

Scale	Injury Type	Classification Criteria
A	Complete	No sensory or motor function preserved in S4-5
B	Sensory Incomplete	Sensory but not motor function is preserved neurological level and extends through S4-5
C	Motor incomplete	Motor function preserved below neurological level with most muscles graded <3*
D	Motor incomplete	Motor function preserved below neurological level with most muscles graded ≥3**
E	Normal	Motor and sensory function is normal.

Adapted from Ditunno et al., 1994
*muscle grade <3 = total paralysis, visible contraction, or active movement without gravity
**muscle grade ≥3 = active movement, against gravity or with some/full resistance

1.2 Secondary injury

1.2.1 Acute phase

Secondary injury commences within minutes of trauma. This acute phase of SCI progresses over the course of 48 hours and is marked by hemorrhage, ischemia, and vascular disruption (Tator and Fehlings, 1991). Consequently, the compromised blood-spinal cord barrier (BSCB) not only allows local, but also peripheral immune cells to respond to necrosis and initiate an inflammatory response. The release of cytotoxic products and inflammatory cytokines further trigger apoptosis in surrounding cells. Acute SCI is also accompanied by a rise in intracellular Na^+ and Ca^{2+} (Agrawal and Fehlings, 1996). Extracellular glutamate is also elevated due to malfunctioning ion channels and transporters (Agrawal et al., 1998; Park et al., 2004). Together, these excitotoxic mechanisms provide another mechanism for axonal degradation and neuronal cell death. Interestingly, injured axons with elevated intracellular Ca^{2+} can recover homeostasis spontaneously and remain in a metastable state for hours, suggesting a self-preservation process exists (Williams et al., 2014).

1.2.2 Subacute phase

The subacute phase of SCI lasts between two days to two weeks post-injury. In this time, inflammation persists via macrophages rather than neutrophils and microglia activation, as in the acute phase. Reports suggest neuron and oligodendrocyte apoptosis contribute to additional cell loss and demyelination (Almad et al., 2011; Liu et al., 1997). Similarly, cell loss can also occur through granular disintegration of axons distal to the injury epicentre, known as Wallerian degeneration. As cell death continues, the formation of a cystic cavity can be seen. Enclosing the lesion core is a layer of reactive astrocytes that form the glial scar. The hypertrophic and proliferative glial cells secrete excess extracellular matrix (ECM) molecules, chemokines, and other inhibitory molecules, forming a physical barrier to regeneration. A fibrotic scar comprised of fibroblasts and pericytes surrounds the glial scar.

1.2.3 Intermediate and chronic phases

The time period between two weeks to six months is termed the intermediate phase of SCI. During the intermediate phase, many of the processes observed in the subacute phase continue to

progress, including the development of the cystic cavity, astroglial scar, and fibrotic scar. Ongoing Wallerian degeneration and maturation of the scar inhibit endogenous regeneration. However, some endogenous axonal sprouting of the corticospinal and reticulospinal tract can be seen. Attempts at endogenous remyelination by both infiltrating Schwann cells and oligodendrocyte precursor cells (OPCs) have also been reported at this stage. Beyond six months after the initial injury, these processes are believed to have stabilized and have reached the chronic phase.

1.3 Preclinical SCI models

Various methods of generating a preclinical model of SCI have been developed, each with their own advantages and disadvantages (see Cheriyan et al., 2014 for review). The three most frequently used models will be described.

1.3.1 Transection

Complete and partial transections involve the use of a scalpel to lesion the spinal cord, separating the rostral and caudal components. Occasionally, longer segments of the spinal cord may also be removed. While this mechanism of injury is extremely rare in clinical SCI populations, this model is often used because of its relative simplicity, and thus, high reproducibility. Typically, the transection model is paired with biomaterial treatments given that distinct borders between tissue and material can be made. In terms of partial transections, targeting damage to specific white and/or grey matter regions can be achieved. This allows researchers to focus on certain sensory or motor pathways, simplifying scientific questions related to recovery and regeneration.

1.3.2 Contusion

To replicate a traumatic injury, a transient and acute impact can be applied to the cord, usually by dropping a weight. However, within this category, the method of classifying and measuring the severity of injury can differ. Some groups prefer changing the inherent weight (g) being dropped on the exposed cord. Other researchers prefer controlling the actual force of impact rather than weight. In any case, this model is believed to provide a more accurate depiction of human SCI pathophysiology. Unfortunately, it is associated with more variability in results than the transection model.

1.3.3 Compression

What both models lack, however, is the compression aspect of SCI. In humans, dislocated and broken bone fragments put compress on the spinal cord until surgery can relieve the pressure. The impactor SCI model has started to address this by leaving the weight sitting on the spinal cord, allowing gravity to exert some pressure on the cord. However, this protocol is not well-established. Small inflatable balloons have also been used to generate injuries. They can be inserted in the epidural space and inflated using air or fluid before deflating after a pre-determined duration. This model is more difficult to perform on smaller rodents and can be quite variable.

1.3.4 Clip-compression

In our lab, we employ a contusion-compression model of SCI. A modified aneurysm clip is used, with the blades placed on the dorsal and ventral surface of the spinal cord. The clip is calibrated to close at a pre-set weight to generate an injury. The initial closure of the clip generates the contusion while the 1 min closure time produces the compression. As a result, this model is believed to be the most faithful to human SCI mechanisms. It has also been used to characterize the vascular components of secondary injury. Unfortunately, its limitations lies in its variability, which is highly dependent on an individual's surgical skills. Nevertheless, we have chosen to use the clip-compression model of SCI for our study to better replicate normal SCI deficits and typical repair and regeneration.

1.4 Current treatments and limitations

Through a better understanding of secondary injury pathophysiology, scientists and clinicians alike can develop targeted treatments to both increase tissue sparing and potentially repair and regeneration of the spinal cord. This would greatly benefit SCI patients as there are currently few treatment options for patients. Peri-injury recommendations include immobilization of patients pre-hospitalization and early transfer to specialized treatment centres. Prior to surgery, patients should undergo comprehensive assessments for physical impairment (Fehlings et al., 2011a), cardiovascular dysfunction, and respiratory insufficiency (Witiw and Fehlings, 2015). Magnetic resonance (MR) imaging is also strongly recommended for acute SCI prognosis.

1.4.1 Early surgical decompression and stabilization

After sustaining trauma, the spinal cord is often compressed by dislocated vertebral bones, and/or ligaments. The goals of decompression surgery are: 1) to relieve the pressure on the spinal cord by removing damaged structures; and 2) to stabilize the spinal column. Since prolonged compression inhibits normal blood perfusion through the cord and exacerbates the secondary injury cascade, it is thought that early intervention provides the best chance at preserving quality of life and subsequent recovery.

Confirming this claim were the results of the Surgical Timing in Acute Spinal Cord Injury Study (STASCIS) trial, a large, multi-centre, cohort study in North America comparing the improvement in neurologic outcome between patients who underwent early (<24 h after injury) or late (≥24 h after injury) surgical decompression (Fehlings et al., 2012). Patients who received early decompressive surgery were more likely to show clinically significant improvements at 6 months follow-up, defined as at least a two-grade improvement in AIS score. The study findings validated preclinical work showing the effectiveness of decompressive surgery in attenuating secondary injury mechanisms is inversely related to the time elapsed since SCI and have since been adapted into the 2017 AOSpine guidelines for acute SCI management (Fehlings et al., 2017a).

1.4.2 Methylprednisolone sodium succinate (MPSS)

Methylprednisolone sodium succinate (MPSS) is the only recognized treatment for acute SCI that has been tested in a large randomized, controlled trial (Vismara et al., 2017). MPSS is a synthetic corticosteroid with potent anti-inflammatory properties useful for preserving blood-spinal cord barrier integrity and spinal cord blood flow. It also serves as a free radical scavenger to mitigate lipid peroxidation (Hall and Braughler, 1982).

Early studies examined the effect of high-dose MPSS as a treatment for acute SCI in a feline model. MPSS-treated animals displayed greater functional recovery and tissue preservation compared to controls (Braughler et al., 1987; Means et al., 1981). Preclinical success and the ongoing need for an SCI treatment helped move MPSS into clinical trial. The National Acute Spinal Cord Injury Study (NASCIS) trials found early administration of MPSS, within 8 of injury, and treatment for 24 to 48 h conferred modest motor recovery in patients both at 6 weeks and 6 months follow-up (Bracken et al., 1997, 1992, 1990). Unfortunately, patients were also

more likely to develop sepsis and severe pneumonia due to their immunosuppression. Numerous groups have also gone on to criticize the NASCIS trials for failure to meet primary endpoints, inconsistencies between participating centres, poor choice of outcome measures, and problematic post hoc statistical analyses (Coleman et al., 2000; Hugenholtz, 2003; Sayer et al., 2006). Consequently, the clinical use of MPSS declined over recent years (Schroeder et al., 2014).

Researchers have tried to replicate the initial studies to provide some clarity regarding the controversial use of MPSS. A systematic review and meta-analysis of both randomized-control trials and observational studies concluded that MPSS did not increase the risk of infections and confers significant short-term motor score improvements when administered within 8 h of an SCI (Evaniew et al., 2016). Patients with incomplete cervical SCI were also more likely to benefit from MPSS treatment (Tsutsumi et al., 2006). In light of these findings, the most recent clinical guidelines have recommended intravenous MPSS for 24 h when initiated within the first 8 h of SCI as a treatment option for patients (Fehlings et al., 2017b).

1.4.3 Haemodynamic maintenance

Since the establishment of guidelines for pre and post-surgical management of acute SCI, with a focus on tissue oxygenation and perfusion, patient survival rates have improved greatly (Witiw and Fehlings, 2015). In clinical practice, this translates to haemodynamic maintenance. Despite initial stable cardiac and pulmonary function, acute SCI patients often develop hypotension, hypoxemia, pulmonary dysfunction and cardiovascular instability between seven to ten days post-injury (Levi et al., 1993). Cardiorespiratory complications not only limit the opportunity for surgical intervention, but it also contributes to secondary damage through spinal cord ischemia. For these reasons, the 2013 American Association of Neurological Surgeons (AANS) and Congress of Neurological Surgeons (CNS) guidelines advocate vigilant monitoring and maintenance of systemic blood pressure (Walters et al., 2013). Clinical practice involves correction of hypotension (systolic blood pressure < 90 mmHg) to maintain mean arterial pressure between 85 and 90 mmHg for the first week after SCI (Ryken et al., 2013). Additionally, to prevent deep venous thrombosis, oxygen saturation should be maintained at >90% and prophylaxis.

When support of mean arterial pressure was first introduced in 2002 (Hadley et al., 2002), the recommendations were primarily founded upon findings small, uncontrolled, and underpowered

studies (Vale et al., 1997) and remained unchanged in the 2013 update (Ahuja et al., 2017; Ulndreaj et al., 2017). However, the published guidelines were validated by more recent retrospective clinical studies, which also suggested vasopressor administration demonstrated neuroprotective potential (Hawryluk et al., 2015).

1.4.4 Rehabilitation

Once patients recover from surgery, a rehabilitation regimen is initiated. Even during hospitalization, a multidisciplinary team of physiotherapists, occupational therapists, and nurses are brought in to assist patients through exercises developed to improve range of motion and muscle strength. The general consensus is that early intervention may shorten a patient's length of stay, prevent secondary complications, and prepare patients for discharge and further rehabilitation programs. This is because activity-based therapy has been shown to promote plasticity of relevant neural circuits and muscle systems (Behrman et al., 2017).

Locomotor or treadmill training is most commonly employed and can be adjusted for weight support (Dobkin et al., 2006). Buehner et al. examined the relationship between locomotor training, AIS score, and various functional outcomes. Although AIS score changes did not reflect recovery following locomotion, they found that treatment improved gait speed to a clinically significant difference that allowed or independent ambulation even after chronic SCI (Buehner et al., 2012). A separate group studied the effects of locomotor training on respiratory function and found that significant improvements in pulmonary measures, including forced vital capacity, forced expiratory volume (1 s), maximum expiratory and inspiratory pressure (Terson de Paleville et al., 2013). Surface electromyography recordings also revealed greater activity of respiratory muscles during voluntary breathing.

Patient heterogeneity is taken into consideration during rehabilitation, but it adds a layer of variability in the care SCI patients receive. Furthermore, rehabilitation protocols and duration vary between hospitals and specialized treatment centres. In order to optimize activity-based therapy, greater effort has been placed on comparing different physiotherapy regimens to identify those that are most effective (Harvey et al., 2016). Clinicians, researchers, and fitness specialists have also worked towards establishing a standardized physical activity guideline for SCI patients, which includes a minimum of 20 min aerobic activities and strength training of major muscle groups twice a week (Ginis et al., 2011).

1.4.5 Limitations and challenges

Thus far, SCI can only be treated acutely by surgical decompression and haemodynamic stabilization. Although MPSS has been approved and indicated for acute SCI, guideline recommendations for its use have been relatively conservative due to its association with increased infection risk. In chronic SCI patients, activity-based training has been shown to promote modest motor and respiratory function via activity-based plasticity. While these findings point to the potential of further recovery, it is unlikely that rehabilitation alone would be sufficient.

Researchers have turned their attention to relieving vascular disruption and inflammation to preserve more tissue and enhance the effects of acute SCI treatments. It is also likely that novel therapies need to address the multifaceted nature of secondary injury mechanisms such as excitotoxicity and reactive astrogliosis to extend the time frame for interventions and promote more drastic changes in sensory and motor function. More importantly, existing treatment options are only able to limit the extent of injury and maximize SCI outcomes rather than promote repair and regeneration of the spinal cord. The central nervous system is notorious for its lack of endogenous repair and regeneration. For this reason, cellular transplantation of stem cells has become an increasingly attractive approach to treating SCI.

1.5 Promising treatments for SCI

The SCI research community has generated much knowledge on novel therapeutic strategies to promote recovery, some of which have translated to clinical trials (Kwon et al., 2005; Ulndreaj et al., 2017). Among the promising candidates are pharmaceutical agents which have demonstrated neuroprotection in preclinical models and have an established safety record in humans for other indications. Other potential treatments that have reached clinical translation have demonstrated robust neurobehavioural improvements in a variety of clinically relevant SCI models.

1.5.1 Riluzole

Developing new pharmaceutical agents for clinical use has proven to be a long arduous process. For this reason, a greater effort has been placed on repurposing FDA-approved drugs for off-label indications. One such example is Riluzole, a benzothiazole Na^+ channel blocker approved for the treatment of amyotrophic lateral sclerosis (ALS). A hallmark of the progressive motor

neuron disease is the demyelination of axons and excitotoxicity (Shaw and Ince, 1997). These pathophysiological mechanisms can also be found in acute SCI. When tested in a transection model of SCI, Riluzole was shown to suppress tail muscle spasticity (Kitzman, 2009). In a separate study using a high cervical spinal hemisection injury, Riluzole was associated with neural preservation, motor recovery, and respiratory recovery (Satkunendrarajah et al., 2016).

Riluzole's preclinical success was enough for researchers to move the drug towards a clinical trial for acute SCI patients. A Phase I/IIA trial was launched to assess the safety and pharmacokinetics of Riluzole in this patient cohort (ClinicalTrials.gov Identifier NCT00876889). A total of 72 patients were recruited for the study with 36 participants receiving treatment and 36 gender, age, and neurological impairment-matched controls. Patients who received Riluzole were administered 50 mg orally every 12 hours for 14 days while controls received the standard of care. Riluzole was shown to be safe and conferred statistically significant AIS motor score improvements, especially in patients with incomplete cervical injury (Grossman et al., 2014). Too few patients (8) and poor sensorimotor tests sensitivity for thoracic recovery were likely the reasons for the lack of improvement seen in thoracic patients (Fehlings et al., 2016).

Accordingly, a follow-up Phase IIB/III trial, Riluzole in Acute SCI Study (RISCIS), to determine safety and efficacy of Riluzole in acute cervical SCI patients began in 2013 (ClinicalTrials.gov Identifier NCT01597518). To date, the multi-centre, randomized, placebo-controlled, double-blind trial has an estimated enrolment of 351 patients. Compared to the earlier study, the dosing regimen was increased to 100 mg two times within the first 24h of injury followed by the original 50 mg twice daily for the remaining two weeks. ISNCSCI scores at 180 days post-injury serve as the primary outcome measure and the study is estimated to be completed by May 2025.

1.5.2 Glibenclamide (Glyburide, DiaBeta)

Glibenclamide (Glyburide) is another FDA-approved drug for diabetes mellitus type 2 being repurposed for acute SCI. Glibenclamide is a sulfonylurea that binds to sulfonylurea receptor 1 (SUR1), an inhibitory regulatory subunit of ATP-sensitive potassium channels (K_{ATP}) (Serrano-Martin et al., 2006), causing membrane depolarization, activation of voltage-gated Ca^{2+} channels, and ultimately insulin release in pancreatic beta cells. SUR1 also regulates Ca^{2+}-activated $[ATP]_i$-sensitive non-specific cation ($NC_{Ca\text{-}ATP}$) channel, whose expression is upregulated in astrocytes and neurons after hypoxia or ischemia (Chen et al., 2003; Chen and

Simard, 2001; Simard et al., 2006). ATP depletion triggers $NC_{Ca\text{-}ATP}$ channel activation and leads to cell depolarization, edema, and oncotic cell death, but glibenclamide was able to mitigate these effects in an ischemic stroke model.

Building upon this work, researchers also examined the expression of SUR1-regulated $NC_{Ca\text{-}ATP}$ channels on endothelial cells in rats with unilateral cervical SCI. They determined that these channels were greatly upregulated in the lesion epicentre, specifically in capillary endothelium (Simard et al., 2007). Compared to the vehicle group, glibenclamide-treated animals demonstrated reduced progressive blood extravasation, decreased lesion volume, and improved functional recovery. It is believed that SUR1-regulated $NC_{Ca\text{-}ATP}$ is a key mechanism underlying progressive haemorrhagic necrosis seen in SCI.

To determine the safety and feasibility of glibenclamide as a neuroprotectant in acute cervical SCI, the Spinal Cord Injury Neuroprotection with Glyburide (SCING) trial (ClinicalTrial.gov Identifier NCT02524379) was initiated in February 2017. 10 patients will be administered glibenclamide orally within 8h of SCI and every 6h following for a total of 12 doses. The trial is projected to conclude in March 2021.

1.5.3 Minocycline

Minocycline is a broad-spectrum synthetic tetracycline antibiotic that is regularly used for the treatment of acne vulgaris. While best known for its anti-microbial properties, minocycline has also been shown to be anti-inflammatory (Yrjänheikki et al., 1999) and anti-apoptotic (M. Chen et al., 2000; Garrido-Mesa et al., 2013) in other neurodegenerative conditions. Its ability to attenuate microglia activation and caspase expression, has also been reproduced in various SCI models along with improvements in long-term hind-limb function (Festoff et al., 2006; Lee et al., 2003; Stirling, 2004; Teng et al., 2004; Wells et al., 2003; Yune et al., 2007).

Combined, these results allowed investigators to proceed with a single-centre, randomized, placebo-controlled, double-blind, Phase I/II trial of intravenous minocycline administration within 12h of SCI for the purpose of dose optimization, safety assessment, and efficacy evaluations (ClinicalTrial.gov Identifier NCT00559494). The trial completed in August 2010 and a total of 52 patients were recruited (minocycline, n = 27; placebo, n = 25). Minocycline was maintained for 7 days and the dosing regimen was generally well-tolerated with only one

minocycline-treated patient experienced a transient elevation in serum liver enzyme levels (Casha et al., 2012). When examining the changes in ISNSCI motor scores at 1-year follow-up, only cervical SCI patient treated with minocycline showed a strong trend towards a 14-point improvement but just failed to reach statistical significance. Nevertheless, there was sufficient evidence to warrant a Phase III study of Minocycline in Acute Spinal Cord Injury (MASC, ClinicalTrial.gov Identifier NCT01828203). With a larger cohort of 248 participants, the investigators aimed to obtain more conclusive motor recovery results between placebo and minocycline-treated patients. The expected completion date was June 2018, but results are pending. Meanwhile, researchers have analyzed cerebrospinal fluid (CSF) samples from their initial Phase II study to find novel biomarkers of SCI to evaluate both injury severity (IL1B, MMP9, HO1) and 1-year prognosis (IL1B, MMP9, CXCL10) (Casha et al., 2018).

1.5.4 Therapeutic hypothermia

Mild to modest systemic hypothermia (32-35^{o}C) is also being pursued as an experimental treatment for neurologic injuries, including SCI. In fact, research into therapeutic hypothermia for stroke and traumatic brain injury date back to the early 1950s (Alkabie and Boileau, 2016). To combat ischemia, a state characterized by a lack of oxygen, ATP, and glucose, therapeutic hypothermia can reduce metabolic demand 6-7% for every 1^{o}C decrease in body temperature (Polderman, 2009). Unfortunately, early clinical studies were poorly designed with small sample sizes and a lack of randomized groups (Dietrich, 2009; Dietrich et al., 2011). Furthermore, control patients did not receive surgical decompression or MPSS as the standard of care. The prolonged protocol for cooling also led to many adverse events including pneumonia and cardiac arrest. Overall, these trials reached inconclusive results and therapeutic hypothermia research was largely abandoned.

In pre-clinical models, hypothermia has been shown to decrease early-gene expression c-fos and excitatory neurotransmitter release to further reduce cell death (Song and Lyden, 2012). From traumatic brain injury (TBI) research, therapeutic hypothermia also stabilized the blood-brain barrier and reduced vasogenic edema (Karibe et al., 1994) by reducing immune cell, inflammatory cytokines, free radicals, and thrombin permeability (Song and Lyden, 2012). These molecular findings were accompanied by long-term behavioural improvements and have reopened the door for clinical trials (Martirosyan et al., 2017).

Drawing from the experience of previous failures, a new multi-centre, randomized, controlled trial began in August 2017 to assess the efficacy of intravenous delivery of modest hypothermia in acute SCI (ClinicalTrial.gov Identifier NCT02991690). Patients will be slowly cooled to 33ºC within 24 h of SCI and maintained for 48 h before normothermia is slowly restored by 0.1ºC. Currently, an estimated 120 patients have been enrolled and the study is expected to be completed by March 2020. The primary outcome measure will be changes in AIS and ASIA motor score between baseline and 12-month follow-up.

1.5.5 Anti-Nogo-A antibody (ATI-355)

Approaching regeneration from a different angle, removing molecular barriers to structural neural plasticity may also be effective in promoting recovery after SCI. Secondary injury mechanisms are known to prevent endogenous regrowth of severed axons, namely arising from components of the glial scar and myelin. In 2 to 6-week-old rats with corticospinal tract (CST) transections, injection of a novel monoclonal antibody (IN-1) against rat spinal cord myelin was able to promote long-distance CST regrowth (Schnell and Schwab, 1990). In slightly older rats, aged 6 to 8-weeks, similar recovery was seen along with specific reflex and locomotor recovery (Bregman et al., 1995). Through greater characterization of IN-1, Nogo-A was found to be the target antigen preventing neurite outgrowth (M. S. Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000; Schweigreiter and Bandtlow, 2006).

Further studies examined a more refined subdural administration of a purified Nogo-A antibody in both a rat thoracic hemisection model (Liebscher et al., 2005) and a monkey unilateral cervical lesion (Freund et al., 2007, 2006). Again, anti-Nogo-A antibody promoted corticospinal axon growth and conferred improved neurological recovery, including manual dexterity in the SCI monkeys. The repeatable results were a strong indication for anti-Nogo-A antibody treatment to test its safety and feasibility in an open-label Phase I trial in SCI patients (ClinicalTrials.gov Identifier NCT00406016, (Zörner and Schwab, 2010). From May 2006 to September 2011, 52 patients began treatment anywhere between 4 to 60 days post-injury and received either continuous intrathecal infusion or multiple intrathecal bolus injections at varying doses (5 mg to 30 mg/2.5 mL/day or 22.5 mg and 45 mg/3mL/4 weeks) (Kucher et al., 2018). 15 patients reported 16 serious adverse events, but only one case was related to anti-Nogo-A antibody's

route of delivery. Generally, the drug was well-tolerated, but the study design limited the investigator's ability to draw conclusions concerning efficacy.

Although the clinical trial demonstrated relative safety to anti-Nogo-A, it also raised concerns of potential off-target effects since the antibody is capable of freely crossing the blood-brain and blood-spinal cord barrier. This was addressed by treating healthy rodents with an intrathecal infusion of anti-Nogo-A antibodies for 2 to 4 weeks (Craveiro et al., 2013). In doing so, researchers confirmed that although the treatment did promote higher expression of neural growth and synaptic markers, these changes were not associated with any cognitive changes or deficits. A secondary question regarding the treatment efficacy was whether future studies should include a broad range of patients varying in time since injury. A follow-up study performed in rodents suggested that the time frame for anti-Nogo-A treatment is restricted to 2 weeks (sub-acute phase) post-injury as longer delays correlated with decreasing effectiveness (Gonzenbach et al., 2012).

1.5.6 BA-210 (VX-210, Cethrin)

Similarly, inactivating enzymes responsible for downstream growth inhibition would work in parallel to neutralizing growth-inhibitory molecules themselves. Rho GTPase mediates actin cytoskeleton remodelling in response to extracellular signals. Upon contact with growth-inhibitory molecules present in the glial scar or on myelin, including Nogo-A, Rho is immediately activated in all neuroglial cells both in the SCI epicentre and penumbra (Fitch and Silver, 2008). Specifically, in neurons, Rho activation is responsible for the collapse of the axon growth cone (Niederöst et al., 2002; Yamashita et al., 2005). Inactivation of Rho by exoenzyme C3 transferase has been shown to promote axon regeneration *in vitro* (Winton et al., 2002). Unfortunately, cells are impermeable to C3 transferase. Alternatively, siRNA knockdown of Rho achieved the same regenerative effect in a dorsal root ganglion model of axon growth (Ahmed et al., 2005), but their potential for off-target effects prevent clinical translation.

To overcome these limitations, researchers modified the C3 transferase sequence to increase permeability. They further eliminated vestigial and protease-sensitive residues (BA-210) for potential clinical use (Lord-Fontaine et al., 2008). Epidural application of BA-210 in a mouse thoracic hemisection model promoted long-distance regeneration of corticospinal neurons, enhanced GAP43 expression in the motor cortex, increased locomotor scores, and improved limb

coordination. Similar improvements in behavioural recovery were also seen in rat thoracic hemisection models.

Capitalizing on these successes, BA-210 (VX-210, Cethrin) was brought to an open-label Phase I/IIa trial in February 2005 to test its safety and tolerability in acute complete cervical and thoracic SCI patients (ClinicalTrial.gov Identifier NCT00500812). A single epidural application of BA-210 at varying doses (0.3 mg to 9 mg) during surgery was tested in 48 patients. No serious adverse events were attributed to drug treatment and preliminary efficacy analysis revealed that cervical SCI patients who received 3 mg of Cethrin showed the greatest AIS motor score improvement, even compared to historical STASCIS data (Fehlings et al., 2011b; McKerracher and Anderson, 2013). This prompted a subsequent multi-centre, randomized, placebo-controlled, double-blind, Phase IIb/III trial to determine the efficacy of high dose (9 mg) VX-210 in patients with acute cervical SCI (Fehlings et al., 2018). An estimated 71 patients were recruited with the study completed in November 2018 (ClinicalTrial.gov Identifier NCT02669849). Given the focus on cervical SCI patients, investigators are predominantly interested in upper extremity motor scores 6 months post-treatment, but results have not yet been published.

1.5.7 Functional and epidural electrical stimulation (FES, EES)

Through activity-dependent plasticity, rehabilitation aims to retain and augment residual volitional movement. However, this relies on patients being able to initiate an action. Fortunately, plasticity can also be driven by applying electrical stimulation below the level of injury, even in chronic SCI patients (Behrman et al., 2017; Mayr et al., 2016). Typically, stimulation needs to be precise both spatially and temporally in order to restore appropriate circuit signalling and this can be achieved by either transcutaneous functional electrical stimulation (FES) or epidural electrical stimulation (EES) in adjunct to rehabilitation.

The non-invasive nature of FES has propelled numerous clinical studies to validate for SCI. Sadowsky et al. Found increased hamstring and quadricep muscle strength and increased quality of life in patients who underwent FES with cycling (2013). When applied to trunk muscles, Triolo et al. claimed FES produced positive changes in stability, posture, and reach (2013). A series of clinical trials assessing FES for upper and lower limb movements have also been completed (ClinicalTrials.gov Identifier, NCT00221117; NCT01208688; NCT00201968; NCT01292811). Although relatively small studies, they observed improvements in grasping

(Kapadia et al., 2013), walking speed, balance, endurance (Kapadia et al., 2014), and bone turnover (Craven et al., 2017).

EES uses the same principles as but allows for greater spatial precision compared to FES. EES over specific spinal cord locations with precise timing can modulate the degree of leg extension and flexion in rats with severe thoracic SCI. Initial studies examined involuntary movements. but the development of a brain-spinal interface (BSI) allowed researchers to examine voluntary movements as well, to enhance relevant task-specific behaviours during rehabilitation (Bonizzato et al., 2018). This was also tested in Rhesus monkeys and improved bipedal locomotion, setting up the framework to evaluate the efficacy of BSI-based gait rehab in paraplegic patients (Capogrosso et al., 2016). Moving to a human population, EES was shown to promote the recovery of volitional leg movements and standing in individuals with chronic clinically complete SCI (Angeli et al., 2014; Rejc et al., 2017a, 2017b). With BSI-controlled lumbosacral EES and rehabilitation, chronically injured SCI patients showed improvements in locomotor performance within 1 week of treatment, while voluntary control of paralyzed muscles without EES could be seen after several months, even outside of rehabilitation settings (Wagner et al., 2018).

1.5.8 Cell therapies

While the previously discussed strategies to treating SCI have focused on allowing the endogenous mechanisms to reach their maximal potential, cell transplantation is not only able to promote endogenous repair, but also replace lost and damaged cells. More importantly, they are also expanding the therapeutic window of SCI towards the subacute and chronic phases of injury. In the case of stem cells, their ability to self-renew, proliferate, and differentiate into specific cell types make them an attractive approach to repairing the injured spinal cord. Much preclinical work has been done to attempt to tease out the best cell type, timing, and dose for transplantation. Although many groups have even moved these studies forward to clinical populations, there is little consensus on the optimal protocol. In the next sections, we will discuss the various cell types that have been tried and tested.

Table 2 Promising treatments for SCI

Treatment	Clinical Trial.gov	Phase	Clinical Reference(s)	Mechanism of Action	Pre-clinical Reference(s)
Riluzole	NCT00876889	I/IIA	Grossman et al., 2014	Decrease excitotoxicity	Kitzman, 2009 Satkunendrarajah et al., 2016
	NCT01597518	IIB/III	Fehlings et al., 2016		
Glibenclamide	NCT02524379	I	N/A	Reduce haemorrhagic necrosis	Chen and Simard, 2001 Chen et al., 2003 Simard et al., 2006
Minocycline	NCT00559494	I/II	Casha et al., 2012	Reduce inflammation, apoptosis	Festoff et al., 2006 Lee et al., 2003 Stirling, 2004 Teng et al., 2004 Wells et al., 2003 Yune et al., 2011
	NCT01828203	III	Casha et al., 2018		
Therapeutic hypothermia	NCT02991690	N/A	N/A	Reduce excitotoxicity, inflammation, vasogenic edema	Karibe et al., 1994 Song and Lyden, 2012
Anti-Nogo-A antibody	NCT00406016	I	Zörner and Schwab, 2010 Kucher et al., 2018)	Axon sprouting	Schnell and Schwab, 1990 Liebscher et al., 2005 Freund et al., 2007, 2006
BA-210	NCT00500812	I/IIa	Fehlings et al., 2011b; McKerracher and Anderson, 2013	Prevent axonal dieback	Winton et al., 2002 Lord-Fontaine et al., 2008
	NCT02669849	IIb/III	Fehlings et al., 2018		
FES	NCT00221117	N/A	Kapadia et al., 2013 Kapadia et al., 2014 Craven et al., 2017	Neuroplasticity	Behrman et al., 2017 Mayr et al., 2016

1.6 Cell types for SCI transplantation

Conceptually, cell replacement strategies seem like a logical approach to repairing the spinal cord after injury, but cells from the central nervous system are not abundantly available or easily isolated/cultured for transplantation. As a result, cell transplantation strategies rely on either peripheral nervous system cells or stem cells.

Stem cells vary in their developmental potential, ranging from totipotency (can generate embryo) to multipotency (restricted lineage), with pluripotency (can generate all cell types) in between. Within each classification, the cell origin and progeny fate further govern whether a cell type is better suited for direct cell replacement, or indirect repair via promotion of a more permissive growth environment.

1.6.1 Embryonic stem cells (ESCs)

Isolated from the inner cell mass of blastocysts are embryonic stem cells (ESCs). Their pluripotent nature makes them highly versatile, but they also have high tumorigenic potential and are not specific enough for SCI treatment. To overcome these challenges, prior to transplantation, ESCs are often differentiated into multipotent cell types found within the nervous system, including neural stem cells (NSCs), oligodendrocyte precursor cells (OPCs), or even terminally differentiated neurons. Still, controversy surrounds the use of ESCs, not only because of their immunogenicity, but also because of their embryonic source.

1.6.2 Induced pluripotent stem cells (iPSCs)

The discovery of key transcription factors (OCT3/4, SOX2, KLF4, and cMYC) have made it possible to reprogram somatic cells to a pluripotent state, known as induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). In doing so, scientists are able to generate a greater source of autologous stem cells without the same contentious ethical discussions of ESCs.

At the same time, iPSCs have raised their own set of concerns. As with ESCs, pluripotency and proliferation pose the risk of tumour formation. iPSCs may also retain epigenetic modifications associated with the parental somatic cells, including methylation, which may hinder reprogramming efficiency (Kim et al., 2010; Ma et al., 2014) and influence tumorigenicity (Iida et al., 2017). Conventional use of viral transduction for reprogramming cells further increase

tumour potential through potential mutagenic integration into the host genome. The development and application of non-viral techniques provide a safer alternative. Pushing iPSCs slightly further along their developmental timeline to more differentiated stem cell subtypes is another option.

1.6.3 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent and stromal in nature and be isolated from both adult and perinatal tissues. Found in bone marrow, adipose tissue, cartilage, umbilical cord, placenta, and perivascular tissue, MSCs can give rise to osteocytes, adipocytes, and chondrocytes. The vastly different sources of MSCs also translate to variable differentiation potencies and have prompted scientists to establish minimal criteria for defining MSCs, including plastic adherence *in vitro*, surface molecule expression of CD105, CD73, CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α, or CD19 and HLA-DR (Dominici et al., 2006), but there is still a need to determine more concise terms for identification (Sipp et al., 2018). Nevertheless, MSCs typically do not differentiate into neuroglial cells, yet they are still of therapeutic interest for SCI because of their relative availability and paracrine effects (Tolar et al., 2010; Wright et al., 2011).

Intravenous infusion of MSCs in preclinical SCI models have resulted in locomotor recovery which was linked to immunomodulatory, anti-apoptotic, and pro-angiogenic effects (Badner et al., 2018, 2016; Morita et al., 2016; Quertainmont et al., 2012; Vawda et al., 2019). Several groups have tested MSCs in Phase I clinical trials, validating the safety of both intrathecal (ClinicalTrials.gov Identifier NCT01909154), intraspinal, and intravenous (Geffner et al., 2008) administration of MSCs. Although initially testing the safety and feasibility of intrathecal MSC injections, investigators also looked a clinical recovery. They found modest but significant improvements in sensory scores, bladder control, bowel control, and sexual function (Vaquero et al., 2016). As a follow up to this study, preliminary efficacy was further assessed in an open-label Phase II study, confirming earlier results (ClinicalTrials.gov Identifier NCT02570932, Vaquero et al., 2017).

1.6.4 Schwann cells (SCs)

Oligodendrocytes are highly susceptible to the hostile SCI microenvironment, especially reactive oxygen species produced by SCI (Almad et al., 2011; Giacci and Fitzgerald, 2018). They undergo necrosis and apoptosis during the acute phase and are seen undergoing apoptosis well

into chronic time points. Multiple studies examining the mechanisms of various neuroprotective strategies have often associated greater white matter sparing with greater neurobehavioural recovery (Chen et al., 2016; Kloos et al., 2005; Scholtes et al., 2011; Tu et al., 2013). For this reason, promoting myelination has been pursued through cell therapies as well.

The peripheral nervous system has demonstrated time and time again superior regenerative potential compared to the central nervous system. Luckily, Schwann cells (SCs) function in parallel to oligodendrocytes in the peripheral nervous system; therefore, they have the capacity to replace them after SCI. Endogenous SCs naturally migrate from the nerve roots towards the lesion to assist in repair (Brook et al., 1998; Nagoshi et al., 2011). They are readily isolated from peripheral nerve fibres and can be purified and expanded for therapeutic use compared to oligodendrocytes (Brockes et al., 1979; Morrissey et al., 1991).

Transplantation studies have demonstrated that exogenous SCs can migrate long distances to remyelinate denuded axons and restore efficient conduction of neural signals (Felts and Smith, 1992; Honmou et al., 1996), even in chronic SCI (Barakat et al., 2005). It believed that SCs mediate repair by modulating the immune response (Pearse et al., 2018), and secreting numerous growth factors, ECM components, and adhesion molecules (Bunge and Wood, 2012). Human SCs have also been tested in rodent models, surviving up to 6 months post-transplantation and promoted greater tissue preservation without tumour formation (Bastidas et al., 2017). Nevertheless, there remain several challenges associated with SC transplantation. Firstly, SCs provoke reactive astrogliosis which may reduce effective integration into the host tissue (Guenard et al., 1994; Williams et al., 2015). Secondly, autologous transplantation requires *in vitro* amplification which is time-consuming. Lastly, studies publishing more convincing functional recovery required adjuvant treatments for increased efficacy (Kanno et al., 2015; Tetzlaff et al., 2011).

Autologous SCs are still promising candidates for SCI treatment and have been translated to a Phase I clinical trial (ClinicalTrials.gov Identifier NCT01739023). 6 patients with acute thoracic SCI were recruited and sural nerve harvests were performed to isolate Schwann cells. Schwann cells were expanded in culture and transplanted within 4 to 9 weeks of SCI and patients were evaluated for 12 months. The study successfully demonstrated the feasibility of harvesting, culturing, and transplanting autologous SCs without a reasonable time frame (Anderson et al.,

2017). Furthermore, the treatment was not associated with any adverse events such as tumour formations or abnormal rates of neuropathic pain, which are major concerns when using SCs.

1.6.5 Olfactory ensheathing cells (OECs)

More distant are olfactory ensheathing cells (OECs), specialized glial cells residing in the olfactory bulb, the transitional zone between the peripheral and central nervous system (Fraher, 1999). During normal cell turnover or after damage to the olfactory nerve or epithelium, endogenous OECs guide the growth of new olfactory receptor neurons through the lamina propria into the nerve layer of the olfactory bulb (Doucette et al., 1983). OECs can be directly isolated from olfactory mucosa biopsies relatively safety and are readily purified and expandable in culture (Féron et al., 1998). These properties make OECs an ideal candidate for autologous central nervous system cell transplantation.

Akin to SCs, OECs are equally able to remyelinate demyelinated axons and promote regeneration across SCI lesions (Barnett and Riddell, 2004; Williams et al., 2004). Not only do OECs secrete lipid vesicles, neurotrophic factors, and ECM molecules, but they also assist in phagocytosis of extracellular debris (Nakhjavan-Shahraki et al., 2018; Roet and Verhaagen, 2014). Compared to SCs, OECs do not cause astrocyte hypertrophy (Lakatos et al., 2000), but they still seem to show the greatest functional benefit when transplanted with additional factors (Tetzlaff et al., 2011). As of now, only one clinical study is registered with the National Institute of Health to treat patients with SCI using autologous OECs (ClinicalTrials.gov Identifier NCT03933072), indicating the need for ongoing preclinical research.

1.6.6 Oligodendrocyte precursor cells (OPCs)

Within the central nervous system, oligodendrocyte precursor cells (OPCs) are the source of oligodendrocytes for endogenous myelination. Perinatal and adult OPCs exist and both populations express NG2 and PDGFα but can be distinguished by the expression of O4, which is only seen on adult OPCs (Levine et al., 2001). Interestingly, OPC populations are predominantly quiescent in adult tissue but will respond to insults by proliferating and differentiating to mature oligodendrocytes (Assinck et al., 2017b; Watanabe et al., 2002). These fate-restricted cells may thus help remyelinate the spinal cord after injury to an extent, limited by growth-inhibitory molecules in the microenvironment (Kawabata et al., 2016). For therapeutic purposes, it is more common to obtain OPCs through the differentiation of ESCs (Faulkner and Keirstead, 2005; Kerr

et al., 2010) or neural stem/progenitor cells (Lü et al., 2010), or by directly reprogrammed somatic cells (Kim et al., 2015).

Most studies have demonstrated proof-of-concept findings for OPC applications using rodent OPCs, but Nistor et al. developed a high-yield protocol for obtaining human OPCs from ESCs that are able to form compact myelin *in vivo* (Alsanie et al., 2013; Nistor et al., 2005). Used in both a thoracic (Keirstead et al., 2005) and cervical (Sharp et al., 2009) contusion SCI model, the human OPCs enhanced remyelination and improved motor function. These studies demonstrate the effectiveness of human ESC-derived OPCs, but before they can be tested in patients, the researchers needed to evaluate preclinical safety in multiple rodent models, included Shiverer/Rag2 mice, SCID/Bg mice, and athymic nude rats. Flow cytometry and immunocytochemistry panels served as quality control for OPC purity and no adverse events were observed after transplantation (Priest et al., 2015). Ectopic cysts were seen at a very low frequency but were not considered a major concern given no clinical symptoms. Findings were repeatable in a cervical SCI model (Manley et al., 2017).

Geron Corporation initiated a Phase I trial of these human ESC-derived OPCs (GRNOPC1) for complete SCI patient in October 2013 (ClinicalTrials.gov Identifier NCT01217008). Although the company planned to enroll 10 patients, the trial was terminated after 4 patient transplantations, citing financial reasons (Scott and Magnus, 2014). Luckily, Asterias Biotherapeutics Inc. stepped in to salvage the trial, this time comparing escalating doses of OPCs (AST-OPC1) in subacute cervical SCI patients (ClinicalTrials.gov Identifier NCT02302157). While the study concluded in December 2018, results have yet to be published. Importantly, caution should be made when eagerly searching for potential trends in clinical recovery as the study was designed to be open-label, without a control group.

1.6.7 Neural stem cells (NSCs)

Earlier in the developmental lineage are neural stem cells (NSCs). Although neural stem cells are defined as having broader potential and greater capacity for self-renewal compared to neural progenitor cells (Gage, 2000), here, the two classes of cells have been grouped together due to the poor distinction throughout literature. In some cases, even NSCs have also been termed precursor cells (Karimi-Abdolrezaee, 2006).

Nevertheless, the rationale in pursuing NSC transplantation stems from the differentiation of NSCs into neurons, astrocytes, and oligodendrocytes. These are the same types of cells that are lost and damaged following spinal cord injury. NSCs, therefore, can be used as a cell replacement therapy for SCI. However, cell replacement is just a small component of this promising cell-based treatment.

Much more research has gone into understanding and optimizing NSCs for SCI. Therefore, we will dedicate a separate section discussing the progress of this cell type.

Table 3 Cell types for transplantation

Cell Type	Therapeutic Source	Clinical Trial.gov	Phase	Mechanisms	Reference(s)
ESC	Inner cell mass	N/A	N/A	Differentiation to multipotent cells	N/A
iPSC	Somatic cells (fibroblast, blood, etc.)	N/A	N/A	Differentiation to multipotent cells	N/A
MSC	Bone marrow, adipose tissue, perinatal tissue	NCT01909154	I	Immunomodulation, anti-apoptotic, pro-angiogenic	Geffner et al., 2008 Vaquero et al., 2016, 2017
		NCT02570932	II		
SC	Peripheral nerve	NCT01739023	I	Remyelination	Anderson et al., 2017
OEC	Olfactory mucosa	NCT03933072	I/II	Remyelination	N/A
OPC	ESCs, iPSCs, NSCs, directly reprogrammed somatic cells	NCT01217008	I	Remyelination	Scott and Magnus, 2014
		NCT02302157	I/IIA		
NSC	ESCs, iPSCs, directly reprogrammed somatic cells	NCT01321333	I/II	Neuroprotection, immunomodulation, angiogenesis, neuroplasticity, remyelination	Curt et al., 2014 Ghobrial et al., 2017 Levi et al., 2017, 2019
		NCT01725880	I/II		
		NCT02163876	II		

1.7 NSCs for SCI

In the adult, NSC populations persist in the subventricular zone (SVZ) and subgranular zone (SGZ) for regular neuron turnover in the olfactory bulb and dentate gyrus of the hippocampus, respectively (Doetsch et al., 1999; Gritti et al., 1996; Johansson et al., 1999; Morshead et al., 1994; Reynolds and Weiss, 1992; Richards et al., 1992). In addition to generating neurons, multipotent NSCs can differentiate into oligodendrocytes and astrocytes. A smaller dormant population of NSCs reside in the ependymal layer lining the central canal of the spinal cord (Hamilton et al., 2009; Weiss et al., 1996). Upon injury, these NSCs become activated and migrate toward the lesion to differentiate and replace cells, predominantly of glial lineage (Beyer and Küry, 2015; Stenudd et al., 2015), to help restrict the extent of injury (Sabelstrom et al., 2013). This natural occurrence, however, is not enough to generate significant neurological recovery, in part due to the small pool of endogenous NSCs. Like OPCs, the preferred therapeutic source of NSCs is differentiated ESCs or iPSCs, or directly reprogrammed cells.

Seeing the regenerative potential of NSCs, multiple research groups have transplanted NSCs in preclinical SCI models to test their efficacy. Making use of a wide range of therapeutic cell sources, including both rodent and human fetal tissue-, ESC-, and iPSC-derived NSCs, and transplanted into various SCI models, such as contusion or transection, most studies have found NSCs enhanced recovery after SCI compared to vehicles (Bonner and Steward, 2015; Mothe and Tator, 2013; Tetzlaff et al., 2011). Remarkably, these effects were typically seen with less than 10% graft survival (Hwang et al., 2016; Parr et al., 2008, 2007; Tarasenko et al., 2007). In some cases, survival was estimated between 20 to 30%, but cell proliferation may have influenced quantification (Okada et al., 2005; Salewski et al., 2015). Of the surviving NSCs, most cells differentiate into astrocytes or oligodendrocytes, with a small percentage of cells turning into neurons (Cummings et al., 2005; Karimi-Abdolrezaee, 2006; Tetzlaff et al., 2011). However, the specific percentages appeared to depend greatly on the derivation of NSCs.

1.7.1 Timing of transplantation

NSC-based therapies are targeted towards the subacute and chronic phases of SCI (Lane et al., 2017). At later time points, the injured spinal cord showed better post-transplant cell survival because the inflammatory processes are believed to have stabilized. Okada et al. found that transplantation time did not change overall behavioural recovery, but only delayed transplanted

animals showed statistically significant recovery long-term (2005). Another study comparing functional improvements between three groups of animals transplanted at different phases of SCI helped validate these findings. Although final BBB scores were similar between all groups, the difference in scores at the time of injection compared to that at the time of sacrifice was greatest in the subacute group (Cheng et al., 2017).

1.7.2 Route and location of injection

The route of NSC administration also varies between studies and may impact the efficacy of the cells. Generally, NSCs are either injected intrathecally or intraspinally. The rationale for using intrathecal implantation relates to the paracrine effects of NSCs. This method is also less invasive compared to intraparenchymal injections. However, intraspinal treatment may make use of more structural, cell-dependent mechanisms which will be discussed in the following segment. A comparison between the two routes of administration revealed that intraspinal implantation increased both grey and white matter sparing, axonal sprouting, and decreased reactive astrogliosis. On the other hand, intrathecal application of NSCs only supported white matter sparing and axonal sprouting, but still facilitated similar motor recovery (Amemori et al., 2015).

To further understand the influence of injection method on efficacy, the location of injection should also be considered. NSC graft survival is greater when transplanted rostral or caudal to the lesion, likely due to the inhibitory factors present within the cystic cavity and epicentre (Piltti et al., 2013). The depth of grafts was also found to change the differentiation profile of NSCs, with more deeply grafted NSCs favouring neuronal phenotypes, whereas NSCs in the periphery, under the pia, presented more astrocytic markers (Yan et al., 2007). In a separate study, 75% of grafted cells differentiated into oligodendrocytes within the white matter, but less than 40% did in the grey matter (Mothe et al., 2008).

1.7.3 Transplantation dosage

Continuing with variables in NSC transplantation, the optimal dose has not yet been defined. Piltti et al. took up this challenge by testing total NSC doses ranging from 2 000 to 100 000 cells per μL. Higher doses of NSCs correlated to decreased cell proliferation 2 weeks post-transplant, but greater cell engraftment 16 weeks post-transplant (Piltti et al., 2015). A subsequent study evaluated alterations in differentiation profile due to NSC dose, with higher doses decreasing the proportion of NSCs converting to oligodendrocytes (Piltti et al., 2017).

1.7.4 Age of transplantation

A much less-studied factor of NSC transplantation is the age of the subject. Most rodent studies use 8 to 10-week-old adult animals. Younger animals may have differential regenerative capacities since their nervous systems are still undergoing development (Semple et al., 2013). Older animals suffer more severe SCI pathophysiology and complications (von Leden et al., 2017), making them more difficult to work with. Although aged mice may experience more extensive damage from SCI, their injured spinal cords appear to be more permissive to NSC treatments, demonstrating greater survival and growth (Takano et al., 2017). In terms of clinical translation, these findings are exciting because of the growing number of elderly SCI patients.

1.8 Mechanisms of NSC-mediated recovery

1.8.1 Neuroprotection

Neuroprotection is thought to occur when a treatment confers an increase in the relative area of healthy-looking tissue in the injury penumbra by dampening secondary injury mechanisms (Assinck et al., 2017a). Following NSC transplantation, grey and white matter sparing is frequently seen and associated with better sensorimotor function. White matter preservation may be attributed to remyelination by differentiated NSCs into oligodendrocytes (Eftekharpour et al., 2007; Karimi-Abdolrezaee, 2006; Plemel et al., 2011). While grey matter changes may be related to NSC-derived neurons, there is greater evidence that NSCs secrete trophic factors, including BDNF, CNTF, GDNF, IGF-1, and NGF, to enhance host cell survival (Hawryluk et al., 2012; Lu et al., 2003).

1.8.2 Immunomodulation

The secretome of NSCs also promotes growth and recovery through non-neural cell types. NSCs have been reported to modulate the immune response by secreting growth factors and anti-inflammatory cytokines (Martino and Pluchino, 2006). Specifically, NSCs can induce phagocytosis, decrease B cell infiltration, and deter classical M1 activation of macrophages, in favour of the more anti-inflammatory M2 phenotype (Cusimano et al., 2012). Of note, it is unclear whether NSCs have a direct or indirect effect on inflammation as NSCs also decrease tissue damage through other means.

1.8.3 Angiogenesis

Vascular pathophysiology in secondary injury is tied to early phases of SCI, but NSCs transplanted at later time points have still managed to demonstrate improvements in angiogenesis. Transcriptome analyses revealed upregulation of angiogenesis-related trophic factor, VEGF, and immunohistochemical analysis supplemented this finding, showing more PECAM-1^{+} blood vessels (Kobayashi et al., 2012). In an independent study by Li et al., NSC transplantation following SCI also induced VEGF expression, along with increased von Willebrand factor-positive cells, and improved limb movement recovery (2014).

1.8.4 Neuroplasticity

Endogenous axon regeneration or sprouting is fairly uncommon after SCI. However, as previously mentioned, NSC-secreted trophic factors may promote endogenous attempts at repairing the injured spinal cord. Apart from the glial scar serving as a barrier to growth, the cystic cavity also poses a challenge as axons prefer a substance over which to grow. NSCs permit growth by serving as a bridge for axons to cross the lesion and have been associated with corticospinal tract regeneration (Kadoya et al., 2016).

Alternatively, damaged circuits can be reconnected by neuronal relays, by which transplanted NSCs differentiate into neurons and serve as the intermediary interneuron between severed axons and dendrites (Assinck et al., 2017a; Bonner and Steward, 2015). In this way, host neurons do not necessarily require long-distance growth. Instead, it relies on graft growth, which is achieved more easily. Unfortunately, this mechanism is unable to explain all forms of recovery, as long-distance graft growth has not been associated with marked behavioural improvements (Kadoya et al., 2016).

For a visual summary of the main secondary injury mechanisms addressed by NSC transplantation, please see Figure 1.

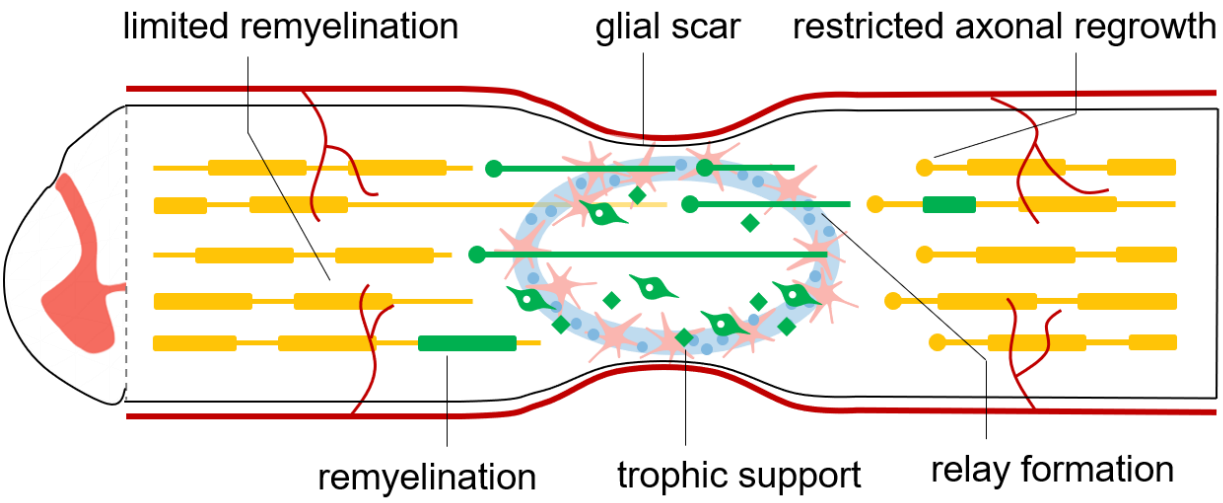


Figure 1 Secondary injury mechanisms targeted by NSC therapy. The chronic injury phase of SCI sees limited remyelination by endogenous oligodendrocytes and infiltrating Schwann cells. The glial scar also surrounds cystic cavity, preventing axon regeneration. NSCs secrete growth factors to promote a more permissive growth environment and differentiate into neurons, oligodendrocytes, and astrocytes to replace lost and damaged cells.

1.9 Moving NSCs towards clinic

1.9.1 Preclinical limitations

Not all SCI studies saw significant functional improvements with NSC transplantation. In some cases, this may be related to the use of complete transection models (Kadoya et al., 2016), which are less clinically relevant. In other cases, this may be related to the use of immunosuppression agents (Pomeshchik et al., 2015). Importantly, very few papers have looked at the chronic effects of NSCs. These studies are not common as they require greater time and resources to keep animals alive, but they are vital to our understanding of human NSCs. The natural developmental timeline of human NSCs requires greater than one or two months to mature. Lu et al. found that prolonged maturation, a minimum of 12 months, was required to observe behavioural recovery (2017).

A secondary consideration is the lack of large mammal models of SCI. While rat and mice may mimic some aspects of secondary pathophysiology, the neuroanatomical organization and size of the spinal cord are not representative of what is seen in humans. A more detailed analysis will be addressed in the following section. Spinal cords of larger animal models, such as pigs and non-human primates, are more comparable to those of humans. Thus, testing NSCs in these models is a key step to help translate preclinical findings to clinical populations (Kwon et al., 2015).

1.9.2 NSCs in clinical trials

Among the better investigated NSC lines for SCI are a human fetal tissue-derived NSCs. Previously Cummings et al. transplanted these human NSCs into immunodeficient NOD-*scid* mice with a T9 contusion injury 9 days post-SCI. NSCs were successfully engrafted into mice and conferred long-term BBB and ladder walk improvements compared to control groups, which was lost after NSC ablation by diphtheria toxin (Cummings et al., 2006, 2005). In terms of translational relevance, treatments that can expand the therapeutic window would be more beneficial. As such, the same NSCs were then tested in a more chronic SCI cohort which replicated similar improvements in locomotor recover, and also showed no increase in allodynia (Salazar et al., 2010). Interestingly, by 16 weeks post-transplantation, NSCs were found to differentiate predominantly into immature neurons (34%) and oligodendrocytes (38%), but very few astrocytes (8%). The remaining population appeared to retain nestin expression, remaining in an NSC-like state.

Nevertheless, these experiments, done in collaboration with StemCell Inc., were convincing enough for the company to proceed with a Phase I/II safety study of these human central nervous system stem cells (HuCNS-SCs) in chronically injured thoracic SCI patients (ClinicalTrials.gov Identifier NCT01321333). Initiated in March 2011 and completed in April 2015, 12 patients were recruited completed and underwent1-year follow-up assessments for adverse events. In parallel, a long-term Phase I/II clinical trial started in November 2012 to determine safety and efficacy of preliminary efficacy of transplanted patients for an additional 4 years after the initial 1-year follow-up (ClinicalTrials.gov Identifier NCT01725880). Unfortunately, this clinical trial was terminated as StemCell Inc. was unable to continue funding such a long-term study. However, an interim analysis presented at an international conference in 2014 suggested HuCNS-SCs were not associated with any adverse events and showed early signs of efficacy for sensory improvement (Curt et al., 2014).

The excitement of these findings prompted a third single-blind, randomized, Phase II trial for HuCNS-SCs, this time, in a chronically injured cervical SCI population (ClinicalTrials.gov Identifier NCT02163876). The first patient was recruited by October 2014, but the study was terminated early yet again due to financial concerns. By May 2016, 31 patients were enrolled. Luckily, the participating investigators still reported their findings, demonstrating the safety free-hand intramedullary transplantation of HuCNS-SC in cervical SCI, similar to thoracic SCI (Ghobrial et al., 2017; Levi et al., 2017). Trends towards motor improvements in HuCNS-SC-treated patients were also seen, but they did not meet predetermined efficacy thresholds, justifying premature trial termination (Levi et al., 2019). While business decisions are not uncommon reasons for clinical trial failures, this study has been the source of additional controversy given that the scientific advisors performing preclinical efficacy studies had warned against the translation of HuCNS-SCs for cervical SCI. Specifically, the NSC line used in the cervical SCI study was not the same line demonstrating efficacy in the thoracic SCI studies. We will discuss the implications of these actions below.

StemCell Inc. is not the only company testing NSCs in clinical trials. Other companies have also attempted testing variations of NSCs for SCI patients, including Neuralstem Inc. Their Phase I safety study of human spinal cord-derived NSCs for chronic SCI began in August 2014 (ClinicalTrials.gov Identifier NCT01772810). A total of 8 patients, (4 cervical, 4 thoracic) were

recruited and monitored over 6 months but formal reports of their findings have yet to be released.

1.9.3 Retrospective clinical considerations

Although several clinical trials have reached completion, we have also reported a series of trials that have been terminated or interrupted due to business decisions, not only. This revisits the challenge in translating preclinical success to clinical success. Modest functional recovery is not enough to convince companies to continue funding research. Part of the problem is the failure in identifying optimal patient cohorts and clinical outcome measures. Cervical SCI patients are ideal for several reasons. They comprise much of the SCI population, and clinical assessments are more sensitive to improvements associated with upper limb deficits. As such, recommendations for the selection of appropriate clinical outcome assessments are being developed (Jones et al., 2018). We have already described one study investigating cervical SCI populations, but it too did not reach completion, suggesting another factor is influencing treatment efficacy.

Conceptually, while it is easy to define what stem cells are, in practice, there are nuanced differences between stem cell populations which often get lost in larger categories. This is particularly important when discussing the proliferative, self-renewal, and differentiation properties of stem cells. From our earlier grouping of neural stem, progenitor, and even precursor cells under the umbrella term “NSCs,” these finer differences are clearly lost. Differences in culture conditions and techniques can easily bias stem cells towards slightly different fates or proliferative potentials (Sun et al., 2011; van der Sanden et al., 2010), and cause a once pure stem cell population to become mixed. If mixed populations are transplanted, it is understandable why variable efficacy may be seen. In some cases, if a research cell line is treated or processed differently from a clinical cell line, there may even be negative impacts on clinical outcomes (Anderson et al., 2017). There is an urgent need to standardize quality control of clinical cell lines and all NSCs for human use must be vetted in rigorous preclinical studies in advance (Anderson and Cummings, 2016).

Summarizing the lessons learned from failed clinical trials, the International Society for Stem Cell Research (ISSCR) put forth a revised set of guidelines that researchers must adhere to before approving future stem cell-based trials (Barker et al., 2018; Daley et al., 2016). In doing

so, the ISSCR hopes to encourage greater oversight and transparency for more fruitful clinical studies.

1.9.4 Combinatorial strategies

By all accounts, research into each cell-based therapy has only shown modest recovery. Although some of the discrepancies may be explained by differences in experimental design (injury model, transplantation time, cell type, cell dose, route of administration) (Tetzlaff et al., 2011), most researchers would also argue that single transplant approaches are not enough to demonstrate drastic clinical improvements (Hachem et al., 2017). For this reason, an increasing number of groups have started to combine multiple cell therapies together (Hosseini et al., 2018; Stewart et al., 2017), or include adjuvant therapies such as biomaterials (Teng et al., 2002; Zweckberger et al., 2016), growth factors (Butenschon et al., 2016; Li et al., 2006), scar-degrading enzymes (Ikegami et al., 2005; Suzuki et al., 2017), and/or rehabilitation (Hwang et al., 2014; Tashiro et al., 2016).

1.10 Neuroanatomy

Understanding the basic neuroanatomy of the spinal cord is vital to developing appropriate therapeutic interventions. Grossly, different levels of the spinal cord innervate distinct muscle groups and receive afferents from distinct sensory domains. At a more detailed level, the spatial distribution of motor and sensory tracts and presence or absence of central pattern generators vary throughout the spinal cord. It is important to distinguish which pathways are impaired following SCI and which pathways mediate distinct aspects of functional recovery.

1.10.1 Corticospinal tract (CST)

Originating from the primary motor cortex and terminating on lower motor neurons in the spinal cord, the corticospinal tract (CST) is the main descending motor tract controlling volitional movements of the trunk and limbs. The upper motor neurons fibres travel through the corona radiata and posterior limb of the internal capsule They continue through the brainstem more medially through the pyramids before the majority of fibres cross the pyramidal decussation. Axons extend along lateral to the dorsal horns and synapse in the ventral horn on to lower motor neurons at their appropriate spinal cord segments.

An MR imaging investigation of acute SCI patients over 12 months found patients had a decrease in cross-sectional spinal cord area and decreases in internal capsule, right cerebral peduncle, and left primary motor cortex volume compared to healthy controls (Freund et al., 2013). These findings were also correlated to functional clinical scores, where a reduced area and volume of CST regions were associated with improvements in spinal cord independence measure (SCIM) score. Similar results were also observed in a separate study, where greater lateral CST damage, as determined by MR image analysis, was correlated with decreased plantarflexion torque (Smith et al., 2018). The CST is also responsible for manual dexterity in adult macaques. After a unilateral SCI, reorganization of CST fibres caudal to the lesion was responsible for restoring some hand function (Nakagawa et al., 2015). Overall, damage to the CST is associated with the loss of motor function seen after SCI and enhancing its plasticity may be a key to improving regenerative therapies (Serradj et al., 2017).

1.10.2 Rubrospinal tract (RST)

Some descending motor tracts are also found in the brainstem. At the level of the rostral midbrain, the rubrospinal tract (RST) emerged from the red nuclei. The fibres immediately cross midline and descend in the lateral funiculi, just lateral to the CST, and synapse either on the inferior olivary complex in the rostral medulla, or the on intermediate region of the ventral horn. Typically, the RST is relatively short and cannot be traced beyond upper cervical segments (Nathan and Smith, 1982). This is not a major motor pathway, rather the RST serves a more modulatory role in humans and non-human primates (Yang et al., 2011).

1.10.3 Vestibulospinal tract (VST)

Reflexes relating to maintaining balance are regulated through the vestibulospinal tract (VST), specifically the lateral portion (McCall et al., 2017). The neurons are found within the lateral vestibular nuclei of the pons and medulla and descend ipsilaterally through the ventral and ventrolateral white matter of the spinal cord, before forming synapses on the ventral gray horn. Clinical evaluation of SCI patients found that poorer Berg Balance Scale (BBS) performance and decreases motor evoked potential (MEP) amplitude was associated with atrophy of the ventrolateral spinal cord, the main region associated with the VST (Barthélemy et al., 2015).

1.10.4 Dorsal column medial lemniscus (DCML)

In terms of sensory pathways, the dorsal column medial lemniscus (DCML) transmits fine touch, vibration, and conscious proprioceptive signals to the sensory cortex. First order neurons are found within the dorsal root ganglion of each spinal nerve and ascend the spinal cord within the dorsal funiculus. Second order neurons are in the nuclei gracilis (lower limbs) or cuneatus (upper limbs) which cross midline and continue rostrally to the ventroposterior nuclei of the thalamus. The final neuron in this pathway terminates on the primary somatosensory cortex. In relation to clinical assessments of sensory deficits following SCI, the AIS or ISNCSCI accounts for the presence of light touch and pin pricks (Kirshblum et al., 2011). Alternatively, sensory evoked potentials may be used to assess DCML function. However, relatively little research has been done on understanding DCML-mediated mechanisms in SCI pathophysiology and recovery.

1.10.5 Spinothalamic tract (STT)

Like the DCML, the first order neurons of the spinothalamic tract (STT) reside in the dorsal root ganglion. The neurons may travel briefly along the tract of Lissauer before synapsing on a secondary neuron within the dorsal horn. These fibres then cross midline and ascend the ventral white matter to synapse on the ventroposterior thalamic nuclei, followed by the sensory cortex. The STT has been linked to pain, temperature, and crude touch. As such, STT damage and sparing has been studied to understand why some SCI patients experience neuropathic pain (Cruz-Almeida et al., 2012).

1.10.6 Spinocerebellar tract (SCT)

Lastly, the spinocerebellar tract (SCT) carries signals pertaining to unconscious proprioception from the spinal cord to the cerebellum. The SCT serves as a feedback loop to assist in the coordination of movements. Multiple divisions of the SCT exist, some travelling ipsilaterally, others travelling contralaterally through the lateral white matter in the spinal cord (Kayalioglu, 2009). While clinical measures specific to SCT damage are not available, some preclinical research has been done to asses the loss and recovery of Clarke's neurons, a region of SCT synapse, in SCI. After a complete T9 transection, fewer identifiable neurons were seen in Clarke's column (Feringa et al., 1985). However, Leung-Wah et al. were able to show that chondroitinase ABC degradation of glial scar components could induce axonal regeneration of Clarke's neurons into a peripheral nerve graft (Yick et al., 2000).

1.10.7 Differences between humans and rodents

Of note, the organization and relative importance of some somatosensory and motor tracts are slightly different between humans and rodents used in preclinical studies. In rodents, the primary and sensory cortices are less well-defined as there are no gyri or sulci. The CST is also found medially, in the inferior portion of the dorsal funiculus, rather than laterally (Nudo and Masterton, 1988; Paxinos and Watson, 2004; Watson et al., 2009). For a better visual depiction of anatomical differences in the spinal cord between species, see Figure 2.

The CST appears to play a less significant role in lower animals as well. The RST larger in rodents and plays a greater role in controlling locomotion. After graded severity clip-compression injuries at the T1 level in a rat model of SCI, a negative linear relationship with motor function was seen, as expected. However, preferential destruction of the large-diameter axons, largely associated with the RST was determined (Fehlings and Tator, 1995). Greater plasticity of the RST neurons compared to those of the CST was also seen in a hemisection injury model (Zörner et al., 2014). Lesioning of the gigantocellular reticular nuclei reverted motor function to levels seen in acute SCI. Unfortunately, early attempts at promoting RST regeneration were unsuccessful (Harvey et al., 2005; Kwon et al., 2004). However, a more refined RST-specific SCI model (Morris and Whishaw, 2016) and multiple rat strains (Mestre et al., 2015) should be used before dispelling RST regeneration as a potential therapeutic target.

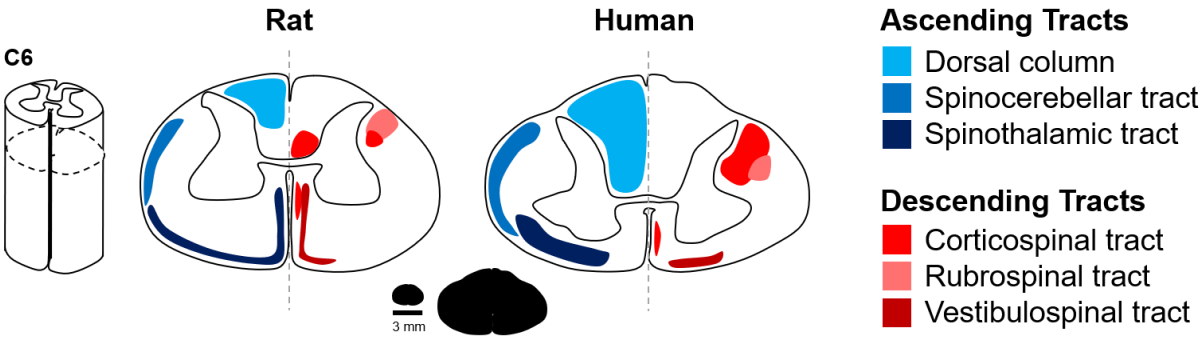


Figure 2 Neuroanatomical differences between rat and human spinal cords. At the C6 level, rat spinal cords measure approximately 3-4 mm in width whereas human spinal cords are approximately 12-13 mm in width. While sensory and motor tracts are found in both species, the organization and distribution throughout the white matter have slight but important differences.

1.11 Tract Tracing

Tracing is necessary to precisely distinguish which neurons are associated with specific sensorimotor pathways and functions. This can occur in two directions: anterograde or retrograde (see Figure 3 for illustration). Once injected into cells, tracer molecules are packaged into vesicles and travel along microtubules via kinesin (anterograde) or dynein (retrograde) (Oztas, 2003). In SCI, anterograde tracing is the preferred method for analyzing axon sprouting and regeneration. Alternatively, retrograde tracing may also be used to trace regenerating neurons by quantifying the number of neurons with regenerated fibres caudal to the lesion. In relation to stem cell transplants, it is unclear whether functional recovery is mediated by their differentiation to neurons. The addition of tracing experiments would identify which motor and/or sensory pathways grafts integrate into to mediate functional recovery.

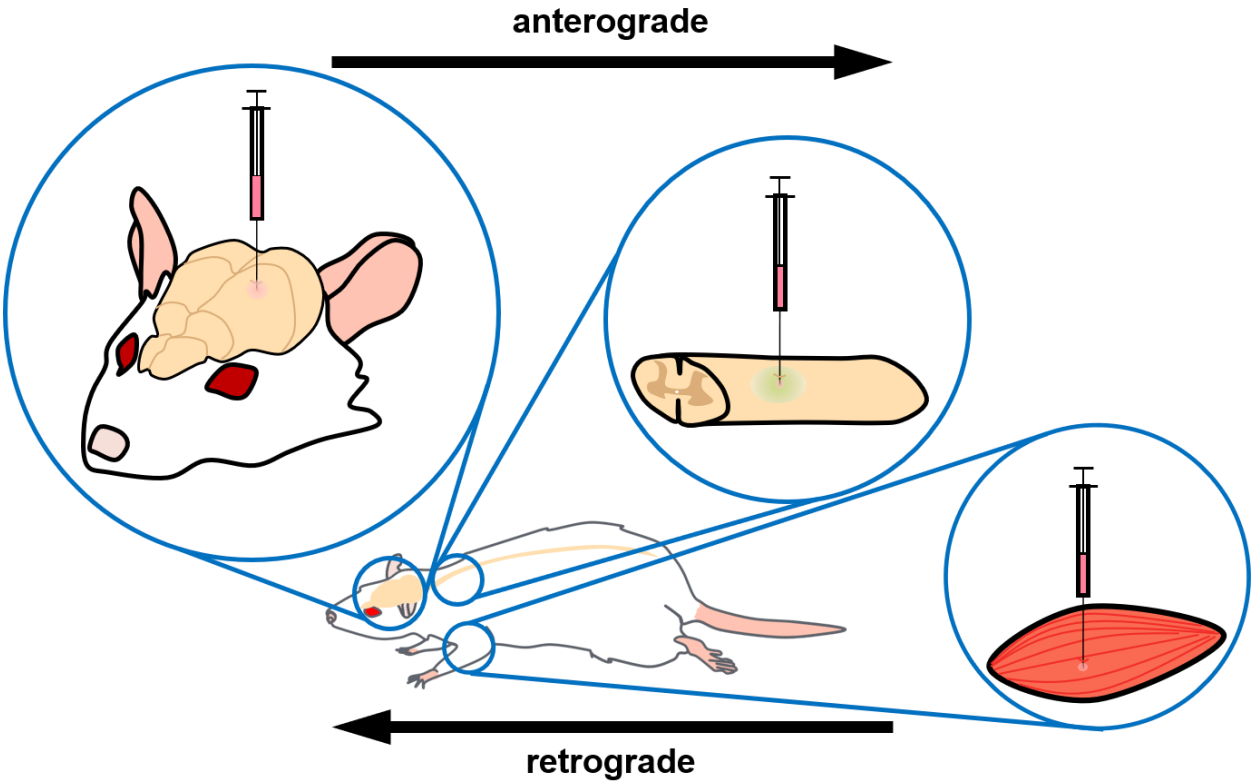


Figure 3 Targets of tract tracing. Injection sites for tract tracing should follow the main sensory and motor pathways. If NSC transplants are centred at the lesion epicentre, anterograde tracing should be done rostral to the injury, even from the sensorimotor cortex. Altneratively, retrograde tracing should begin caudal to the injury, such as from the innervated muscles targets. Ideally, tracing should be seen in the grafted NSCs.

1.11.1 Non-viral

Beginning with non-viral tracers, horseradish peroxidase (HRP) is a 44 kDa enzyme isolated from *Cochlearia armoracia* that has demonstrated bidirectional intra-axonal tracing potential (Aschoff and Schönitzer, 1982; van der Want et al., 1997). More recently, HRP is used as a conjugate to other tracer molecules, such as biotinylated dextran amine (BDA), a commonly used in tracing studies for their sensitivity and ability to permanently label targets. BDA can be found in a range of molecular weights, with 3 kDa and 10 kDa being the most popular for retrograde and anterograde tracing, respectively (Lazarov, 2013; Reiner et al., 2000). While diaminobenzidine (DAB) labelling can assist with visualization of traced pathways, fluorescent dextran amines also exist. If retrograde tracing is desired, Fluorogold is an ideal tracer (Bilsland and Schiavo, 2009; Catapano et al., 2008).

While many of these conventional tracing techniques are useful, they are largely non-trans-synaptic. Some trans-synaptic tracers do exist, such as cholera toxin B (Lai et al., 2015). However, non-viral tracers are unable to replicate, causing signal dilution after each synaptic step. The poor detection of tracers may be misinterpreted as a lack of synaptic connectivity. Therefore, non-viral trans-synaptic tracers are still used in combination with each other (Lanciego et al., 1998) or viral trans-synaptic to verify neural connections (Arriaga et al., 2015).

1.11.2 Viral

When trans-synaptic tracing is desired, viral tracing is preferred. The most utilized viruses are herpes simplex virus (HSV) and pseudorabies virus (PRV). Multiple strains of HSV exist, some of which move in the anterograde direction, while others move in the retrograde direction (Norgren and N. Lehman, 1998). On the other hand, PRV is a robust retrograde tracer (Enquist, 2002). Researchers have taken advantage of the inherent affinity of these neurotropic viruses to move along neural pathways (Callaway, 2008). Additionally, viral replication occurs within the host neurons before infecting adjacent synaptic connection, amplifying the signal and avoiding dilution seen in non-viral methods. Viruses have been genetically modified to express different reporter genes for detection (Banfield et al., 2003; Elliott and O'Hare, 1999; McGovern et al., 2012; Smith et al., 2000). Further genetic modification of viruses can increase the specificity of tracing (Beier and Cepko, 2012; Kim et al., 2016; Lo and Anderson, 2011; Wall et al., 2010; Zeng et al., 2017).

In order to use viral tracing effectively, it is equally important to consider their limitations. While many of their limitations can be overcome by modifying promoters or adding a Cre-lox system, this does not change the toxicity of the technique (Sun et al., 2019). Taking the best aspects of non-viral and viral tracers—safety, trans-synaptic properties, and potential for genetic modification—we describe two molecular tracers below.

1.11.3 Wheat germ agglutinin (WGA)

Wheat germ agglutinin (WGA) is a plant lectin routinely used in neuroanatomical studies to map various pathways. Originally isolated from *Tricticum vulgaris*, the cysteine-rich WGA protein measures 18 kD binds specifically to N-acetyl-D-glucosamine and N-acetylneuraminic acid (sialic acid) residues in the carbohydrate moiety of glycoproteins and glycolipids (Nagata and Burger, 1972; Ruda and Coulter, 1982). These targets are present on the extracellular surface of plasma membranes of neurons, making WGA an effective tracer for neural systems (Wood et al., 1981).

Neurons uptake intact WGA and transport the molecule bidirectionally to axons and dendrites, but predominantly in the anterograde direction (Huh et al., 2010). Once in the cell, WGA is localized to the neuron soma and proximal dendrites (Fabian and Coulter, 1984). More specifically, when motor neurons in the facial nucleus were traced by WGA, the signal appeared punctate, with WGA found within lysozymes and multivesicular bodies, but not in surrounding glial cells (Ruda and Coulter, 1982). Despite intensive efforts to understand WGA transport, researchers are only able to show that WGA undergoes endocytosis, travels slowly on actin filaments but quickly on microtubules (Liu et al., 2011). WGA may stay within the cell undergoing this cyclic process between actin and microtubule transport, or it may undergo exocytosis.

Injections of WGA into well-known neural pathways have labelled both first- and second-order neurons, demonstrating trans-synaptic transport (Huh et al., 2010). Often, neuronal projections can be labelled within 2 days of injection (Levy et al., 2015). In one case, WGA could be detected in the lumbosacral spinal cord 6 h post-injection into the cerebellum (Reeber et al., 2011). In addition to its relative ease of use, commercial availability, and lack of toxicity, WGA can also be conjugated for easier visualization. When fused to other proteins such as HRP (Hoshino et al., 2010; Itaya and Van Hoesen, 1982; Takeuchi et al., 2009), GFP (Oh et al., 2011),

DsRed (Sugita, 2005), or Alexa fluorophores (Goshgarian and Buttry, 2014; Reeber et al., 2011), WGA maintains trans-synaptic activity and presents more robust signals. WGA can further be paired with Cre-recombinase systems to restrict tracing in discrete neuronal (Damak et al., 2008; Libbrecht et al., 2017).

1.11.4 Tetanus toxin fragment C (TTC)

Looking at retrograde tracers, tetanus toxin fragment C (TTC) displays retrograde axonal and trans-synaptic transport (Coen et al., 1997). Tetanus toxin (TeTx) itself is a potent inhibitor of neurotransmitter release produced by *Clostridium tetani*. Early studies involving intramuscular TeTx injections in rats showed localization of the toxin to motor neurons and afferent synaptic terminals (Schwab and Thoenen, 1976). TeTx was limited to neurons, with surrounding glial cells being unlabeled. These findings revealed the retrograde nature of TeTx but its applications as a tracer were not yet clear, especially since animals succumb to clinical tetanus and death within 14 h of treatment.

The 50 kDa heavy chain of tetanus toxin, also known as TTC, was obtained by protease digestion (Bizzini et al., 1977; Neubauer and Helting, 1981). Researchers determined that this domain was responsible for cell-binding and toxin internalization (Calvo et al., 2012). Once bound, the toxin could be internalized by clathrin-dependent mechanisms, independent of synaptic vesicle recycling (Deinhardt et al., 2006). To avoid unnecessary use of a dangerous neurotoxin, a non-toxic recombinant TTC protein was produced and reported to possess identical properties and TTC achieved by papain cleavage of TeTx (Halpern et al., 1990). When injected into paravertebral ganglia of pigeons, TTC could be found in both myelination and unmyelinated sympathetic preganglionic axons, moving retrogradely at an approximate rate of 10 mm/h (Cabot et al., 1991). This opened the doors for using TTC as a carrier for other proteins.

Coen et al. generated a hybrid *lac*Z-TTC protein which could be internalized in neurons, distributed throughout the cytosol, both *in vitro* and *in vivo* (1997). By expressing β-galactosidase with TTC, the authors were able to visualize retrograde trans-synaptic transport more easily by applying X-gal to reveal a blue stain. This demonstrated proof-of-concept that fusion of recombinant TTC to a visual marker is an effective strategy to map neural networks. Similarly, cytoplasmic staining of cultures Neuro2A cells and primary rodent spinal neurons after treatment with a GFP-TTC protein confirmed the feasibility of this technique (Kissa et al.,

2002). Importantly, TTC was only co-labelled in $MAP2^+$ neurons, but not $GFAP^+$ or $O4^+$ glial cells. When Neuro2A cells were transfected with GFP-TTC and grafted into the caudate-putamen, tracing of the globus pallidus, substantia nigra, and motor cortex, all known input regions, was observed 5 days later. GFP-TTC could also be expressed in transgenic mice to map neural circuits throughout development (Maskos et al., 2002).

1.11.5 Use of tracing in other neurological conditions

NSC transplantation is not unique to SCI. In fact, NSCs have been transplanted in other traumatic injuries and neurodegenerative diseases. In all its uses, the integration of NSCs into host networks may be necessary to confer functional benefits. Tracing may be used to help elucidate these pathways. Similarly, neurodevelopmental studies would also benefit from tracing techniques to delineate the formation of distinct sensory and motor pathways. This topic will be discussed in more detail in the conclusion.

1.12 Rationale

To summarize, human NSCs have proven to be a promising regenerative SCI treatment. Their self-renewal, proliferative, and neuroglial potential allow them to replace lost cells and support endogenous repair by the secretion of trophic factors. Preclinical studies have repeatedly demonstrated neurobehavioural improvements associated with NSC transplants. Unfortunately, these findings have not been as successful when translated to clinical populations. A deeper look into NSC-mediated repair mechanisms may help us understand how to optimize NSC therapies.

Our lab has previously shown the importance of NSC differentiation to oligodendrocyte in remyelinating denuded axons. However, more studies are demonstrating graft differentiation to neurons that form extensive axon growth. To probe whether neuronal relay is indeed a valid mechanism by which NSCs confer functional recovery, we believe trans-synaptic tracing is necessary to demonstrate definite graft integration. Unfortunately, conventional tracing techniques pose numerous challenges. Non-viral tracers are easier and safer to use, but they are largely non-trans-synaptic. Viral tracers, while trans-synaptic, can only show monosynaptic tracing due to neurotoxicity with prolonged exposure. More importantly, both strategies require separate injections sites to NSC transplants, resulting in poor overlap of the tracer with engrafted cells. To overcome this feat, we aimed to engineer self-tracing NSCs to express both antero- and retrograde trans-synaptic tracers.

1.12.1 Overarching hypothesis

Self-tracing NSCs will maintain typical NSC characteristics and will be effective in tracing their synaptic connections once differentiated into neurons.

1.12.2 Specific aims

Aim 1: Generate a monoclonal line of self-tracing NSCs by subcloning, transfecting, and sorting a stable line of hiPSC-NSCs.

Aim 2: Characterize self-tracing NSCs for self-renewal, proliferation, and multipotency.

Aim 3: Validate the trans-synaptic tracing potential of self-tracing NSCs *in vitro*.

A summary of the experimental approach can be found in Figure 4.

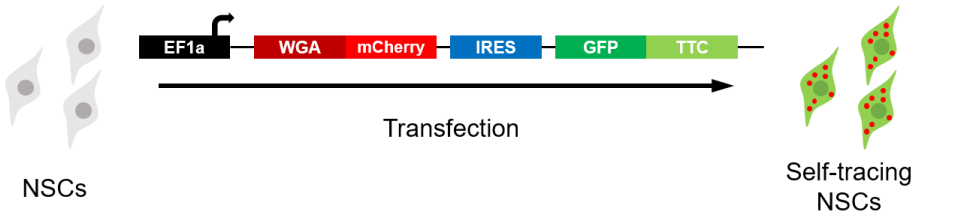
A

Aim 1	
Generation of self-tracing NSC	• Gibson subcloning • Transfection (electroporation) • FACS

Aim 2	
In vitro NSC characterization	• Neurosphere assay • RT-PCR • Immunohistochemistry
In vitro NSC differentiation	• RT-PCR • Immunohistochemistry • Whole-cell recording

Aim 3		
Validate trans-synaptic tracing	*In vitro* co-culture	• Immunohistochemistry
	In vivo transplant	• Immunohistochemistry

B



	Graft	Host		
		Anterograde	Retrograde	Untraced
mCherry	+	+	(+)	–
GFP	+	–	+	–
Human	+	–	–	–

C

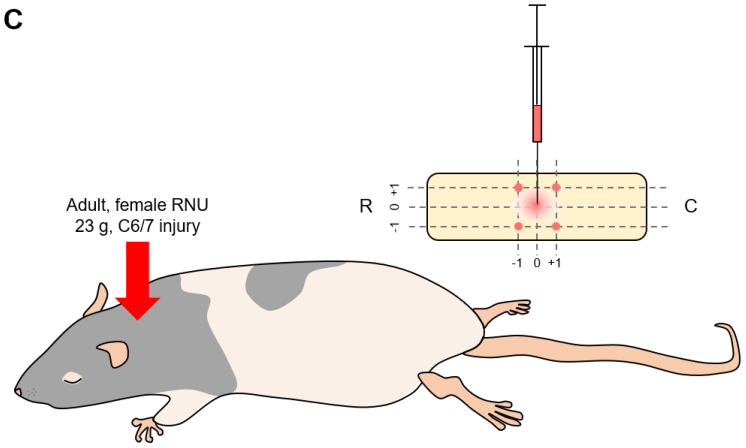


Figure 4 Overview of experimental design. (A) A summary of the experimental aims, beginning with the generation of self-tracing NSCs and ending with a proof-of-concept tracing experiment. (B) Human iPSC-NSCs will be transfected with a bicistronic vector to express anterograde WGA-mCherry and retrograde GFP-TTC tracers. When transplanted into a rodent SCI model, grafts can be distinguished by strong dual-positive fluorescence and human-specific markers. In endogenous host cells, mCherry and GFP signal should be distinguishable based on the direction of tracing. (C) Atyhmic RNU rats will undergo sham or 23 g C6/7 contusion-compression injury. At 8 weeks post-SCI, animals will receive self-tracing NSCs at 5 injections sites (200 000 cells/µL, 2 µL in cavity, 1.5 uL per additional site).

Chapter 2
Materials and Methods

2 Materials and methods

2.1 Genetically engineering self-tracing NSCs

2.1.1 Plasmid subcloning

An EF1a::WGA-mCherry-IRES plasmid was previously generated by M.K. and C.S.A (see Supplementary Figure 1). GFP-TTC was subcloned immediately downstream of the IRES sequence by Gibson assembly to generate a bicistronic vector. Restriction enzymes and adapter fragments used are found in Tables 4,5. Gibson assembly was performed using the Gibson Assembly Ultra Kit (SGIDNA). Electrocompetent *E. coli* were transformed (Bacterial Program 6, Amaxa Nucleofector) with the assembled product and plated at 30^{o}C under ampicillin and/or kanamycin selection. Colonies were picked and grown for Miniprep (GeneAid) to screen correctly assembled products. Positive colonies were further expanded for Maxiprep (GeneAid).

To verify the size of undigested plasmids and digested fragments, 10 ng samples (or 10 μg for extraction) were run on a 0.8% agarose gel with SYBR Safe (10 000X, Thermo Scientific). A 1 kb plus DNA ladder was used to measure the length of fragments and the gel was imaged using the BIORAD imager. If specific fragments were needed for additional subcloning, the small fragment was briefly visualized using a UV transilluminator box and excised from the gel using a sterile blade. Gel fragments were immediately processed using the Monarch Gel Extraction Kit (New England Biolabs) to obtain DNA.

Table 4 Restriction Enzymes

Restriction Enzyme	Recognition Site	Associated Fragment(s)
KflI	GG*GWCCC CCCWG*GG	GFP-TTC Adapter TTC-PolyA gblock
MluI	A*CGCGT TGCGC*A	TTC-PolyA gblock TTC-WPRE Adapter
SacI	GAGCT*C C*TCGAG	hH11(5)-EF1a Adapter
SalI	G*TCGAC CAGCT*G	hH11(5)-EF1a Adapter polyA-hH11(3) Adapter
SgsI	GG*CGCGCC CCGCGC*GG	polyA-hH11(3) Adapter

Table 5 EF1a::WGA-mCherry-IRES-GFP-TTC

Fragment	Sequence
hH11(5)-EF1a Adapter*	TCTTGGAAATTACACTATAGATATCCTGGTCCGCGGTCGACGGATCG ATCGCGGCATATCATGCGACTGATCGATCGCAATTATATCAGACCCG CGATCGCGACGTCGATCGATGCATGCAAATGTAGCTAACTATCATCG CACGGGCAGTGTGCATGCATCAACATGATCATATCGCGGTAGCTAC GATGCTCGATCGATGCTAGCAGCTCCCGTGAGGCGTGCTTGTCAAT GCGGTAAGTGTCACTGAT
GFP-TTC Adapter	CAGACCACACTCAGATGAACACTCCAATCGGAGGGGGTCCCGTACA CGTACCCGAGTATCATCACCTGACCGTCTGGACATCGTTTGGAAAAG ACCCTGACGACGATGAAACTGATCATCTCAACATTGTGGAAGTGATC AAGGCGGTGGACTTGGAAACATACCGGATGAAG AACCTGGACTGCT GGGTTGACAAC
TTC-PolyA gblock	ATGAAGAACCTGGACTGCTGGGTTGACAACGAAGAGGACATCGACG TGATCCTGAAGAAGTCCACCATCCTGAATCTGGACATCAACAACGAC ATTAACATCGACATCAGCGGCTTCAACAGCAGCGTGATCACATACCC CGACGCTCAGCTGGTGCCTGGCATCAATGGAAAGGCCATCCACCTG GTCAACAATGAGTCCAGCGAAGTGATCGTGCACAAGGCCATGGACA TCGAGTACAACGATATGTTCAACAACTTCACCGTGTCCTTTTGGCTGC GGGTGCCCAAAGTGTCTGCCAGCCACCTGGAACAGTACGGCACCAA CGAGTACAGCATCATCTCCAGCATGAAGAAGCACAGCCTGAGCAT CGGAAGCGGCTGGAGCGTTAGCCTGAAGGGCAACAATCTGATCTGG ACCCTGAAGGACTCTGCCGGCGAAGTGCGGCAGATCACCTTTAGGG ACCTGCCTGACAAGTTCAACGCCTACCTGGCCAACAAATGGGTGTTC ATCACCATCACCAACGACCGGCTGAGCAGCGCCAACCTGTACATCAA TGGGGTGCTGATGGGCAGCGCCGAGATCACAGGACTGGGAGCTATC AGAGAGGACAACAACATCACCCTGAAACTGGACCGGTGCAACAA CAACAATCAGTACGTGTCCATCGACAAGTTTCGGATCTTCTGCAAGG CTCTGAACCCCAAAGAGATCGAGAAGCTGTATACCAGCTACCTGAGC ATTACCTTTCTGCGGGACTTCTGGGGCAACCCTCTGAGATACGACAC CGAGTACTATCTGATCCCCGTGGCCTCCAGCAGCAAGGACGTGCAG CTGAAGAACATCACCGACTACATGTACCTGACAAACGCCCCTAGCTA CACCAACGGAAAGCTGAACATCTACTACCGGCGGCTGTACAACGGC CTGAAGTTCATCATCAAGCGGTACACCCCTAACAACGAGATCGACAG CTTCGTGAAGTCCGGCGACTTTATCAAGCTGTACGTCAGCTATAACA ACAACGAGCACATCGTGGGCTACCCCAAGGACGGCAACGCCTTTAA CAACCTGGACAGAATCCTGAGAGTGGGCTACAACGCCCCTGGCATC CCTCTGTACAAAAAGATGGAAGCCGTGAAGCTGCGGGACCTGAAAA CCTACAGCGTCCAGCTGAAACTGTACGACGACAAGAACGCCTCTCTG GGCCTCGTGGGCACACACAATGGCCAGATCGGCAACGACCCCAACC GGGACATTCTGATCGCCAGCAACTGGTACTTCAACCACCTGAAG GATAAGATCCTGGGCTGCGATTGGTACTTCGTGCCCACCGATGAAG GCTGGACCAACGATTAATAGTGATTCAGACATGATAAGATACATTGAT GAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATT TGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGC AATAAACAAGTTGTCGACCATGGAACGAGGTTAAGGTCTCTAAA
TTC-WPRE Adapter	CGACCATGGAACGAGGTTAAGGTCTCTAAAGCACAGTACATGGACAG ATATCCACGTAGATATTACGAGACGATTGAGACGATAGCCAGTATG ACGATAGACGATATGGATAGACCATGCTTACCCGGTAAGCCAATCG GGTATACACGGTCGTC
polyA-hH11(3) Adapter*	TTTCATAGGTCACTTACATATATCAATGGGTCTGTTTCTGGTACACAT TCGGCATCGACGATGTAATCGATCGATCGATGCTGGCTATCTATAGC ACAGACAGACAATATGGGCGCTTACGCGATATATGCGGATTCGACG ACTTCGATCGACGCATATGTATCACATACATGCGCGCATCGACTCTG ATGCATCGTCGCCAACTCGTACACGCGCCAGGCCTCATATGATCGAT TAATTAACCGGTCCTA

* hH11 Adapters were only used to generate CRISPR plasmids

2.1.2 NSC transfection

hiPSC-NSCs were previously generated by M.K. In brief, BC1 cells, a hiPSC line derived from $CD34^+$ bone marrow cells of a 46 y.o. male, were obtained from NIH (Johns Hopkins University School of Medicine) and underwent dual SMAD inhibition for neural induction (Chambers et al., 2009). The resultant hiPSC-NSCs were maintained in culture in Serum Free Media (SFM) composed of: DMEM/F12 media with GlutaMAX (Gibco), Hormone Mix (see Supplementary Table 1), B27 Supplement minus Vitamin A (Gibco), EGF (20 ng/mL, Wisent), FGF2 (20 ng/mL, Wisent), Heparin (2 μg/mL, BioShop), and Penicillin-Streptomycin (Gibco). See Supplementary Table 2 for details.

In a 10 cm culture dish coated with Geltrex (Gibco), hiPSCs were grown to 70-80% confluency. TrypLE (Gibco) was added to and cells were left at room temperature for 2-5min until lifted. 2 volume equivalents of 10% FBS was added to inhibit further enzymatic activity. Cells were transferred to a new tube, pelleted, and resuspended in Nucleofector solution (Lonza) along with 20 μg plasmids. The cell suspension was then transferred to a 2 mm-gap cuvette for electroporation (A-033, Amaxa Nucleofector). To reduce cell death, SFM with Rho/ROCK inhibitor (10 μM, Y-27632) was added to the cuvette and cells were gently transferred to a new Geltrex-coated plate. Additional SFM was added as necessary. Media changes were performed regularly to wash out non-viable cells. Expression of mCherry and GFP was monitored over the course of 2 weeks. Cells then underwent fluorescent activated cell sorting (FACS) into coated 96-well plates.

2.1.3 Fluorescent activated cell sorting (FACS)

Once cells achieve 70-80% confluency, NSCs were ready for FACS. Geltrex-coated 96-well plates were prepared with 100 μL of SFM with Rho/ROCK inhibitor to reduce cell death. Cells were sorted on purity mode through a 100 μm nozzle. SFM change was done the following day to remove the Rho/ROCK inhibitor and Ca^{2+} from the sheath fluid. Subsequent media changes were done once a week until fluorescence could be visualized. 2 weeks post-FACS NSCs were screened for GFP and mCherry expression, with only strong dual-positive cells being passaged for expansion.

2.2 Stem cell characterization of self-tracing NSCs

After genetically modifying the NSCs, the canonical phenotypes needed to be verified to ensure that the self-tracing cells remained NSCs.

2.2.1 Neurosphere assay

To assess self-renewal and proliferative potential, self-tracing NSCs and control GFP-NSCs were plated at a clonal density of 10 cell/μL in a final volume of 500 μL SFM in uncoated 24-well suspension plates (Starstedt). Neurospheres grew undisturbed for 7 days. The original description of the assay suggested that the average size of neurospheres capable of propagating were, on average, 150 μm in diameter (Rietze and Reynolds, 2006; Weiss et al., 1996). Our lab has previously demonstrated that spheres greater than 50 μm are also ready for passaging. For this reason, neurospheres greater than 50 μm in diameter were quantified. Just prior to imaging, the contents of each well were transferred to a Geltrex-coated dish, incubated for 30 min, and fixed with 4% PFA. Duplicate wells also prepared for propagating NSCs into secondary and tertiary neurospheres by mechanically dissociating cells, re-plating them, and allowing them to grow for an additional 7 days.

2.2.2 RT-PCR of NSC markers

GFP and self-tracing NSCs were plated on 6-well plates coated with Geltrex at 1 x 10^6 cells per well. 2 days post-plating, cells were washed 3 times with 1X PBS. NSCs were lifted as using a cell scraper and pelleted for mRNA extraction (Exiqon). A Nanodrop spectrophotometer was used to evaluate mRNA concentration and purity. cDNA was synthesized (Bioline) and diluted to 1 ng/μL for RT-PCR using TaqMan probes (Applied Biosciences): GAPDH (Hs02786624_g1), MSI1 (Hs01045894_m1), NES (Hs04187831_g1), NOTCH1 (Hs01062014_m1), PAX6 (Hs01088114_m1), SOX1 (Hs01057642_s1), SOX2 (Hs01053049_s1), VIM (Hs00958111_m1). Markers were chosen based on key transcription factors, signalling molecules, and cytoskeletal proteins associated with NSCs (Oikari et al., 2016). Within each NSC group, cells were plated from different passage numbers to obtain an average fold change value (n=5). Samples were run in triplicates with values normalized to GAPDH.

2.2.3 Immunocytochemistry (ICC) of nestin

Self-tracing NSCs were plated on Geltrex-coated glass coverslips in 24-well plates, approximately 10 mm in diameter. Cells were fixed in their respective wells for 20 min at room temperature with an 8% PFA in 30% sucrose solution, added at a 1:1 ratio of media to fixative for a final solution of 4% PFA. Coverslips were washed three times with 1X PBS (10 min). For intracellular staining, cells were subjected to a solution of 0.1% Triton in PBS to permeabilize the cell membrane and placed on ice for 3 min. Blocking of non-specific antigen binding was achieved using a solution of 5% BSA for 1 h at room temperature. The primary antibody for NES (1:200) was diluted in blocking solution and applied to the coverslips for overnight incubation at 4ºC. WGA and TTC antibodies were also used to enhance tracer signal. Following three washes (15 min) of 1X PBS, secondary antibodies Alexa Fluor 488, 568, 647 (1:1000) and/or Q565 (1:100) were applied with DAPI (1:1000) for 1 h at room temperature. An additional three washes with 1X PBS, Mowiol was used to mount the coverslips on to slides. Slides were allowed to dry overnight at room temperature and transferred to 4ºC for long-term storage.

2.3 Differentiation to neuroglial lineage

Another key feature of NSCs is their ability to differentiate into neurons, astrocytes, and oligodendrocytes. The general differentiation profile was first assessed by treating cells with spinal cord homogenate to ensure that self-tracing NSCs were not abnormally biased towards specific fates by RT-PCR. This was further examined by inducing single lineage differentiation and assessing immunocytochemistry. All cells were plated on Geltrex-coated 10 mm glass coverslips and placed into 24-well plates.

2.3.1 RT-PCR of NSC, neuron, astrocyte, and oligodendrocyte markers

GFP and self-tracing NSCs were plated on 6-well plates coated with Geltrex at 1 x 10^6 cells per well. 100 μg/mL of spinal cord homogenate (extraction described in section 2.6.2) from naïve or 8 week-injured SCI animals was used to treat cells for 1 week, with regular media changes performed every other day. NSCs cultured in SFM were plated as controls. After 1 week of treatment, NSCs were lifted as using a cell scraper and pelleted for mRNA extraction (Exiqon). A Nanodrop spectrophotometer was used to evaluate mRNA concentration and purity. cDNA was synthesized (Bioline) and diluted to 1 ng/μL for RT-PCR using TaqMan probes (Applied

Biosciences): GAPDH (Hs02786624_g1), NES (Hs04187831_g1), SOX1 (Hs01057642_s1), SOX2 (Hs01053049_s1), DLG4 (Hs01555373_m1), GAP43 (Hs00967138_m1), TUBB3 (Hs00801390_s1), AQP4 (Hs00242342_m1), GFAP (Hs00909233_m1), APC (Hs01568269_m1), and PDGFRA (Hs00998018_m1). Samples were run in triplicates with values normalized to GAPDH.

2.3.2 NSCs to neurons

Neural induction media (NIM) replaced SFM to initiate differentiation of NSCs towards neurons. NIM was composed of Neural Basal Media (Gibco), B27 Supplement with Vitamin A (Gibco), N2 Supplement (Gibco), hBDNF (10 ng/mL, PeproTech), dibutyryl cAMP (1 nM, Sigma), Penicillin-Streptomycin (Gibco). 1 week post-induction, maintenance of neural differentiation and further maturation of neurons were achieved by modifying NIM to Neural Maintenance Media (NMM) by removing hBDNF and cAMP. Media compositions can be found in Supplementary Tables 3,4.

2.3.3 NSCs to astrocytes

To differentiation NSCs to astrocytes, SFM was replaced with 1% FBS. Differentiation was evident as early as 1 week post-induction.

2.3.4 NSCs to oligodendrocytes

Oligodendrocyte differentiation by NSCs required a longer protocol with sequential treatment EC23, a retinoic acid receptor (RAR) activator (0.1 μM, Stem Cell Technologies), Shh (1 μM, PeproTech) PDGFAA (20 ng/mL, PeproTech), and T3 (30 ng/mL, Sigma Aldrich). NSCs were cultured in DMEM/F12 supplemented with N2 supplement and treated with EC23 for 3 days. From days 2-7, Shh was added. On day 7, PDGFAA was added for 7 days. Maturation of oligodendrocytes was promoted using T3 for a minimum of 7 days but could be prolonged.

2.3.5 Whole-cell patch clamp recording

Patch clamp recordings were performed by A.V. A coverslip was placed in a perfused chamber mounted on Nikon E600FN microscope equipped with infrared differential interference contrast optics (IR-DIC) and an epifluorescence system that included a computer-controlled excitation wavelength switcher Lambda DG-4 (Sutter Instruments) and a high-resolution digital CCD

camera (ORCA ER C4742-95-12, Hamamatsu). Images were acquired using NIS Elements (AR3.10, Nikon) software and analyzed using ImageJ version 1.52 (Wayne Rasband, NIH).

The chamber was perfused at a rate of 0.5 ml/min with a solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$. 10 HEPES, 15 D-glucose and 3 Na-pyruvate, pH adjusted to 7.2 with NaOH (osmolarity ~295 mOsm, measured with a freezing point Advanced Micro-Osmometer).

The cells were visualized and selected for patch clamp recording using IR-DIC and fluorescence for GFP and mCherry using appropriate filter cubes (EX480/40x, BS 505, EM510 for GFP and EX535/50x, BS 565, EM610/75 for mCherry).

The patch pipettes were pulled on a laser-based programmable pipette puller (model P-2000, Sutter Instruments) and filled with a solution containing (in mM): 130 K gluconate, 10 HEPES, 1 EGTA, 0.1 $CaCl_2$, 2 MgATP, 5 NaCl and 0.3 Na-GTP (pH 7.2 adjusted with KOH, osmolarity ~280 mOsm) and had resistances between 3 and 5 MΩ. The patch pipette solution was supplemented with 0.5 mM Lucifer Yellow (LY, dilithium salt, MW 457). LY fluorescence was recorded using the same filter used for GFP. Due to a much brighter LY signal in cells injected with LY during whole-cell recording, cell processes could be visualized and cells easily recognizable after formaldehyde-fixation of the coverslips post-recording.

The cells were approached with a patch pipette using an MPC-385 Motorized Micromanipulator (Sutter Instruments). The pipette was pressurized to 10 mm Hg during approaching the cells, and a gigaseal (2-4 GΩ) was formed by releasing the pressure after establishing a close contact of the pipette tip with the cell membrane. A whole-cell mode of recording was established by mouth-applied brief negative pressure to rupture the membrane and establish a direct connection between the pipette interior and the cell cytoplasm. The gigaseal formation was monitored using the Membrane Test feature of pClamp10 software (Axon Instruments, Molecular Devices).

The signals, low-pass filtered at 10 kHz and sampled at 20 kHz, were recorded with a computer-controlled MultiClamp 700B amplifier, digitized with Digidata 1440 and processed/analyzed using pClamp10 software (all from Molecular Devices). Both voltage clamp and current clamp recording (current clamp). Capacitance compensation and bridge balance were performed using the automatic functions of the MultiClamp 700B amplifier. To record Na^+ and K^+ currents, in voltage-clamp mode the cells were held at -80 mV, and voltage was stepwise changed in 10 mV

increments between -110 mV and +40 mV. For testing the ability of cells to generate action potentials, in current clamp mode, the membrane potential was held at around -70 mV, and 50 and 250 ms-long current pulses were applied to depolarize the membrane to action potential threshold levels, and up to 0 mV or higher when no action potentials were detected. To detect spontaneous synaptic activity, 2 minute-long continuous ("gap-free") voltage clamp and current clamp recordings were performed in each cell, with membrane voltage held at around -70 mV.

All recordings were done at room temperature.

2.3.6 ICC of neuroglial markers

Cells were fixed in their respective wells for 20 min at room temperature with an 8% PFA in 30% sucrose solution, added at a 1:1 ratio of media to fixative for a final solution of 4% PFA. Coverslips were washed three times with 1X PBS (10 min). For intracellular staining, cells were subjected to a solution of 0.1% Triton in PBS to permeabilize the cell membrane and placed on ice for 3 min. Blocking of non-specific antigen binding was achieved using a solution of 5% BSA for 1 h at room temperature. Primary antibodies for TUBB3, MAP2, and GFAP (Supplementary Table 5) were diluted in blocking solution and applied to the coverslips for overnight incubation at 4^{o}C. WGA and TTC antibodies were also used to enhance tracer signal. Following three washes (15 min) of 1X PBS, secondary antibodies Alexa Fluor 488, 568, 647 (1:1000) and/or Q565 (1:100) were applied with DAPI (1:1000) for 1 h at room temperature. An additional three washes with 1X PBS, Mowiol was used to mount the coverslips on to slides. Slides were allowed to dry overnight at room temperature and transferred to 4^{o}C for long-term storage.

2.4 Co-culture

2.4.1 Neuro device (Campenot chamber)

18 mm glass coverslips were coated with PLO (10 μg/mL, Sigma) and Laminin (5 μg/mL, Millipore) with 3 washes with sterile H_2O after each step. The Neuro Device was UV sterilized prior to placement over a gently dried coated coverslip. NIM was applied to one well with gentle suction applied to the adjacent well to generate a directional flow of media. The process was repeated for the bottom wells. The assembled device was placed into 12-well plates, with empty wells filled with sterile H_2O to minimize evaporation. Self-tracing NSCs (pB only) were cultured

in SFM as previously described and resuspended at a density of 1 x 10^6 cells/mL in NIM. With some gentle suction applied, self-tracing NSCs were added to the top well in 20 μL increments until cells were seen to fill the chamber. Additional NIM was used to top up wells. Cells were left at 37ºC, 5% CO_2 and media changes were performed every other day. 2 weeks post-plating, primary rat cortical neurons were plated into the bottom chamber and NIM was replaced with NMM to allow for neuronal maturation. Cells were co-cultured for an additional 2 weeks with regular media changes. Prior to patch clamp recordings or immunocytochemistry (ICC), the Neuro Device was carefully removed with fine forceps.

2.4.2 Primary rat cortical neuron isolation

Primary cortical neurons were obtained from E17-19 rat embryos (Charles River). Following euthanasia by CO_2, pups were removed from the pregnant rat and decapitated under aseptic conditions. The heads were placed into a 10 cm dish containing cold HBSS (Gibco) for dissection. Brains were removed from the skull and the meninges were discarded using fine forceps. The cortices were further dissected from the diencephalon and transferred to a centrifuge tube for 3 washes with fresh HBSS. TrypLE was used to digest the tissue at 37ºC for 10 min. After the addition of 10% FBS, the cortices were gently triturated and passed through a 40 μm cell strainer with excess 10% FBS. Cells were centrifuged at 1200 rpm for 4 min, the supernatant was aspirated, and the pellet was resuspended in NMM. Cells were diluted to the desired density and plated as necessary.

2.5 Animals

All experimental procedures involving the use of animals received approval from the Animal Care Committee at the University Health Network and under the supervision of clinical veterinarians. Experiments were performed in accordance with the policies found in the Guide to the Care and Use of Experimental Animals as per the Canadian Council of Animal Care.

Female Rowett Nude (RNU) rats (Charles River) were received at 6 weeks-old and housed in pairs with *ad libitum* and water under 12h light-dark cycles. Rats were allowed to reach 8 to 10 weeks-old prior to experimentation. Animals were closely monitored for weight, hydration, and bladder function by animal care staff and clinical veterinarians throughout the study. Humane euthanasia endpoints were determined *a priori* and were adhered to.

2.5.1 Clip contusion-compression SCI model

Adult female RNU rats aged 8 to 10 weeks-old were anaesthetized with 5% isoflurane for rapid induction carried by 1:1 NO_2-O_2. Isoflurane was reduced to 2% for maintenance. Animals were placed on heating pads to maintain constant body temperature. An additional gauze bolster was placed under the torso to achieve optimal access to the laminae and spinal cord during surgery. The upper backs of the rats were shaved and cleaned with 2 wipes each of 70% ethanol and betadine. To ensure post-operative analgesia, animals were administered buprenorphine (0.05 mg/kg) subcutaneously. 10 mL saline was also administered to ensure hydration.

A No.15 blade was used to create a midline dorsal skin incision from the occipital protuberance to the prominent T2 spinous process. A second incision was made along ligamentum nuchae to reach the laminae while minimizing bleeding. The muscle layers were retracted to expose the paraspinal muscles and the attachments of the spinal and deep muscles were laterally removed from the vertebrae to the medial edge of the articular facets by scraping with a scalpel. Ligamentum flavum was removed from the rostral end of C6 to the caudal end of C7 using the tip of the scalpel the tip of the scalpel. The laminae were removed using angled offset bone nippers. At this point, the procedure is considered a C6/7 laminectomy, and this was performed on sham animals.

To create space for the extradural passage of the modified aneurysm clip, a blunted dissection hook was inserted in the space between the C6/7 spinal roots bilaterally. The bottom blade of the clip was passed along the ventral surface of the spinal cord. To generate the injury, the clip was applied quickly with a closing force of 23 g and the compression was sustained for 1 min. Puncture of the dura or spinal cord resulted in euthanasia and exclusion from the study.

Following removal of the clip, the retractors were released. Muscles were closed using 3-0 nylon sutures along the midline and the skin was closed using Michel suture clips. Animals were removed from isoflurane and allowed to recover under a heat lamp in a clean cage with softened food and water on the floor.

2.5.2 Post-operative care

Amoxicillin (Apo-Amoxi) or amoxicillin with clavulanic acid (Clavamox) was supplemented in the drinking water 3 days prior to injury and maintained until endpoint. Animals were

administered buprenorphine (0.05 mg/kg) twice daily for 3 days and meloxicam (1 mg/kg) once daily for 5 days post-injury. Injured rats were also given 10 mL saline twice daily for 7 days. Additional saline injections and nutritional support were administered as necessary until animals returned to >80% pre-operative weight. Bladders were expressed manually three times per day until reflexive control and micturition were restored.

2.5.3 Cell-transplantation

Self-tracing NSCs (pB only) and GFP-NSCs were cultured in SFM as previously described (see 2.1.2. NSC Transfection). Prior to transplantation, cells were collected in SFM at a density of 200 000 cells/μL. Cells were kept on ice until they were ready for use. Each sample could only be used for up to 2 animals (2 h max).

2 weeks or 8 weeks post-injury, animals underwent similar surgical procedures as described above (see 2.5.1 Clip contusion-compression SCI model) with slight modifications. The scar tissue was carefully removed by working from the rostral and caudal ends of the C6/7 region. Using the tip of a 25G needle and a pair of fine forceps, the dura was opened. Animals were transplanted at 5 sites: directly in the epicentre cavity (1), 1 mm rostral and 1mm lateral to the epicentre (bilateral, 2), 1 mm caudal and 1 mm lateral to the epicentre (bilateral, 2). 2 μL of the cell suspension was infused into the epicentre while 1 μL was used in the penumbra. A 10 μL Hamilton syringe and microinjector pump was used the regulate the volume and rate of cell transplantation (1 μL/min). Injection needles were left to dwell for 2 minutes after injection to prevent backflow. The meniscus was checked before each transplant and the syringe was thoroughly flushed with saline between each animal to ensure there was no blockage.

2.5.4 HSV tracing

Naïve female Wistar rats were used to test viral tracing. HSV-GFP tracing was performed by anaesthetizing the animal as described in section 2.5.1. The animal's head was secured to the stereotaxic frame. A superficial incision was made along midline over the skull of the animal using a No. 15 blade. Bregma and the sagittal suture were used as landmarks. A small fragment of the skull on the right side was carefully removed using a dental drill bit (2 mm lateral to midline by 1.5 mm caudal to bregma). A 10 μL Hamilton syringe was used to withdraw the viral solution. 6 injections were performed at -0.5 mm, -1.0 mm, and -1.5 mm to Bregma, 1 mm and 2 mm lateral to midline. 1 μL of HSV was injected per solution at a rate of 0.1 μL/min, regulated

by a microinjector pump. The needle was left in place for 2 min to prevent backflow. Animals were kept for 5 days prior to sacrifice.

2.5.5 PRV tracing

PRV152 tracing of the flexor digitorum profundus (FDP) muscle was performed as follows. A small superficial incision of the forearm was made. Scissors were used to blunt-dissect the tissue to further expose the underlying fascia and muscles and identify the four main muscle groups. Once the muscle was located, a small drop of saline over the opening to was used to ensure the tissue was moist. Meanwhile, 10 μL of PRV was pipetted on to a cold glass surface covered in parafilm. A 10 μL Hamilton syringe and needle were used to withdraw the viral solution. A finer 35G needle was then swapped for injection. 3 to 4 free-hand injections angled parallel to the muscle fibres were used for a total injection volume of 6 μL. Injections were done slowly while carefully withdrawing the needle from the muscle to ensure minimal reflux. The fascia was closed using 10-0 nylon sutures and the skin incision was closed with 5-0 sutures. After addressing the wound, the rat was placed into a new clean cage where it was housed for 96 h until perfusion.

2.6 Tissue processing

2.6.1 Spinal cord homogenate

Animals were deeply anesthetized with isoflurane and transcardially perfused with 180 mL ice-cold 0.1 M PBS at 8 weeks post-SCI. At the time of tissue removal, the injury level was reconfirmed. The injury epicentre (0.5 mm) was collected into a microcentrifuge tube with 1 mL ice-cold DMEM/F12. Samples could be processed immediately or at a later date if flash froze in liquid N_2.

A blue-tip homogenizer was used to gently extract protein from the spinal cord sample. Samples were then centrifuged at 1200 g for 4 min to pellet unwanted tissue. The supernatant was sterilized through a 0.45 μm filter and quantified by microBCA. The resultant protein extract was used at a final concentration of 100 μg/mL

2.6.2 Immunohistochemistry (IHC)

Animals underwent anesthesia and transcardial perfusion with 180 mL ice-cold 0.1 M PBS and 4% PFA at 2 weeks or 8 weeks post-SCI. The brain and spinal cord were removed from the animal and post-fixed 10% sucrose in 4% PFA solution overnight. The following day, the tissue was washed with 0.1 M PBS and cryoprotected in 30% sucrose in PBS for 48 h.

A section of the spinal cord measuring 2.5 cm in length surrounding the lesion epicentre was embedded in M1 embedding matrix and stored at -80^{o}C. Brains were embedded in Tissue-Tec Optimal Cutting Temperature and stored at -80^{o}C. Tissues were sectioned using a cryostat in 30 μm thick sections in either transverse or longitudinal sections.

For immunohistochemistry, slides were thawed, dried, and rehydrated with 1X PBS. Non-specific binding was prevented using a 1h incubation with blocking solution (5% milk, 1% BSA, 0.3% Triton) at room temperature. Primary antibodies for WGA and TTC were diluted in blocking solution and incubated overnight at 4^{o}C. Three 15 min washes with PBS were performed before applying secondary antibodies Alexa Fluor 488 and 568 with DAPI (1:1000) for 1h at room temperature. Upon an additional three PBS washes, Mowiol was applied to the slides to mount coverslip. Slides were allowed to dry at room temperature overnight and transferred to 4^{o}C for long-term storage.

2.6.3 Statistics

Quantitative data are expressed as mean ± SEM. Although sample sizes were relatively small, repetition of experiments revealed a normal distribution of the data, favouring the use of a parametric statistical test. Therefore, differences between groups were assessed by one- and two-way analysis of variance (ANOVA) with Tukey's post hoc test to correct for multiple comparisons ($p < 0.05$). Data were analyzed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, US, http://www.graphpad.com).

Chapter 3
Results

3 Results

3.1 Generation of monoclonal self-tracing NSCs

3.1.1 Subcloning of bicistronic vector

The vector was based on a plasmid obtained from Addgene (#55632), which contained EF1a::mCherry-IRES-Cre in an AAV backbone (Fenno et al., 2014). M.K. and C.S.A. used various methods to: 1) switch the backbone from an AAV to piggyBac (pB) backbone, previously obtained from the Nagy Lab; and 2) insert a WGA as a fusion protein upstream to mCherry and GFP downstream to IRES, replacing Cre recombinase. P.C. then performed Gibson subcloning to insert TTC as a fusion protein upstream of GFP, following the sequence found in the original paper published by Kissa et. al (2002). After growing and isolating individual bacterial colonies transformed with the plasmid, diagnostic RE cuts were performed to verify successful assembly (Figure 5). Similar procedures were used to assemble a CRISPR-Cas9 variant of the self-tracing plasmid, targeting the human Hipp11 locus (hH11) (Figure 6). Samples of plasmid DNA were also sent for sequencing to confirm sequence fidelity (see Supplementary Figures 2,3 for full sequence).

3.1.2 Expression of WGA-mCherry and GFP-TTC in NSCs

Transfected NSCs were screened for mCherry and GFP expression as a proxy for WGA and TTC expression. Initially, approximately 1% of a 10 cm plate displayed dual-positive expression of the markers. After FACS, individual cells were expanded over 2 weeks before the second round of fluorescent screening was performed using an epifluorescent microscope. Only cells maintaining a ubiquitous expression of both mCherry and TTC were passaged for further growth (Figure 7). After multiple passages, the pB line of self-tracing NSCs retained strong tracer expression but the signal weakened in the hH11 line (Supplementary Figure 4).

3.2 Characterization of self-tracing NSCs

3.2.1 Self-tracing NSCs retain self-renewal and proliferative properties

WGA TTC (pB) and WGA TTC (hH11) self-tracing NSCs were tested for self-renewal and proliferative potential by neurosphere assay against a control line of GFP-expressing NSCs, previously generated and tested in our lab (Figure 8). The number of primary neurospheres greater than 50 μm were comparable between all NSC lines (GFP: 36.0 ± 2.8; hH11: 33.6 ± 4.7; pB: 32.8 ± 3.0). Similar numbers of neurospheres were also observed after the first (GFP: 32.0 ± 1.8; hH11: 36.0 ± 2.5; pB: 33.6 ± 2.0) and second passages (GFP: 37.6 ± 3.0; hH11: 36.8 ± 2.3; pB: 35.2 ± 3.4). Self-tracing NSCs also maintained tracer expression (Figure 8B).

3.2.2 Self-tracing NSCs express typical NSC markers

To validate that NSC phenotypes of self-tracing cells were reflected at the molecular level, RT-PCR was used to evaluate expression levels of known NSC markers in self-tracing NSCs post-FACS expansion against GFP NSCs (Figure 9A). Generally, self-tracing (pB) NSCs showed similar expression levels of all markers to control cells. However, self-tracing (hH11) NSCs showed a down-regulation of most markers, but they were not statistically (one-way ANOVA, Tukey's post hoc, $p < 0.05$) different, except for MSI1 (GFP: 1.00 ± 0.04; hH11: 0.67 ± 0.06). Moreover, ICC confirmed positive staining of nestin in self-tracing NSCs.

3.3 Differentiation of self-tracing NSCs

3.3.1 Profiling self-tracing NSC differentiation *in vitro* by RT-PCR

Self-tracing NSCs were treated with either naïve or 8 week-injured spinal cord homogenates to induce differentiation of cells into their neuroglial fate over the course of 7 days. GFP NSCs were also used as controls, and relative expression changes were compared to SFM-cultured NSCs. Once mRNA was isolated and converted to cDNA, RT-PCR of NSC, neuron, astrocyte, and oligodendrocyte markers was performed (Figure 10). Fold changes have been $\log_2$ transformed.

Compared to SFM-cultured NSCs, GFP and both lines of self-tracing NSCs demonstrated a slight increase in NSC markers, namely SOX1 and SOX2 (two-way ANOVA, Tukey's post hoc, $p < 0.001$) when treated with naïve homogenate, but a decrease in expression with SCI

homogenate. This suggests factors within the SCI microenvironment possibly promotes possible differentiation of NSCs to neuroglial fates.

Upon naïve spinal cord homogenate treatment to NSCs, a slight increase in neuron marker expression was seen, which was most statistically significant for the marker DLG4 (GFP: 1.39 ± 0.26; hH11: 0.59 ± 0.12; pB: 1.18 ± 0.19, $p < 0.01$), a gene involved in recruiting receptors, ion channels, and signaling proteins to post-synaptic sites. Similar patterns of upregulation in a naïve spinal cord microenvironment was seen with both astrocyte and oligodendrocyte markers. The greatest upregulation was seen with AQP4 (GFP: 11.70 ± 0.09; hH11: 11.08 ± 0.06; pB: 11.07 ± 0.09, $p < 0.0001$), a gene encoding water channels, localized to astrocytic endfeet. Oligodendrocyte marker, such as APC, showed slightly less drastic upregulation (GFP: 2.84 ± 0.27; hH11: 1.67 ± 0.08; pB: 1.89 ± 0.08, $p < 0.0001$). These findings hint that NSCs are more likely to differentiate into astrocytes and oligodendrocytes than neurons.

After NSCs were treated with SCI homogenate for 1 week, a decrease in neuron markers was detected. Interestingly, GAP43 expression (GFP: -1.32 ± 0.12; hH11: -2.64 ± 0.09; pB: -2.21 ± 0.11) was not only lower than in naïve conditions (GFP: 0.59 ± 0.42; hH11: -0.70 ± 0.07; pB: -0.28 ± 0.08, $p < 0.0001$), but also in control SFM conditions (GFP: N/A; hH11: -0.87 ± 0.04; pB: -1.64 ± 0.14, $p < 0.01$). Astrocyte and oligodendrocyte markers were generally only slightly elevated compared to control conditions. Only AQP4 expression remained elevated (GFP: 7.65 ± 0.10; hH11: 5.44 ± 0.15; pB: 6.73 ± 0.11, $p < 0.0001$). Astrocytes appear to be the predominant fate of NSCs in an SCI microenvironment.

3.3.2 *In vitro* induction of NSC differentiation to neurons

Given that the RT-PCR results for neural differentiation of NSCs were not convincing, we wanted to confirm the neural potential of self-tracing NSCs by inducing differentiation *in vitro*. After 1 week in culture with NIM, self-tracing NSCs stained positive for immature neuron marker, TUBB3. Switching to NMM for an additional week in culture, self-tracing NSCs expressed MAP2, a cytoskeletal marker found in more mature neurons (Figure 11B). When plated within Campenot chambers, neurons could be seen growing axons up to 800 μm (Figure 11E). Self-tracing NSC differentiation to astrocytes and oligodendrocytes were also studied by *in vitro* differentiation. Self-tracing NSCs displayed robust GFAP expression, typical of astrocytes.

However, due to several incidents of bacterial contamination in shared incubators, long-term self-tracing NSC differentiation to oligodendrocytes could not be confirmed.

3.3.3 Self-tracing NSC-derived neurons are electrically functional

While ICC hinted as successful neural differentiation, functional neurons are required not only for integration after transplantation, but also for transmission of trans-synaptic tracers. As such, whole-cell patch clamp recording was used to evaluate the electrical phenotype of self-tracing NSC-derived neurons. NSCs were differentiated for a total of 4 weeks prior to recording, in hopes of studying a more mature neuron. Neurons were identified by dual-positive expression of mCherry and GFP under epifluorescence. Once a seal was formed, voltage clamp recordings were used to probe the presence of Na^+ and K^+ currents. As seen in Figure 12C, the sharp downward peak points to the presence of active Na^+ channels for fast Na^+ influx. This was followed by a slower and more prolonged outward K^+ current, typical of neurons. In addition to observing ion conductance, the firing of action potentials by current clamp recording was also seen. Together, the hallmarks of functional neurons suggested that self-tracing neurons would have the potential to integrate into host circuitry and trace their synaptic connections.

3.4 Validation of self-tracing NSCs

3.4.1 *In vitro* proof-of-concept by self-tracing NSC co-culture with primary rat cortical neurons

Once the functional activity of self-tracing NSC-derived neurons was confirmed, activity-dependent trans-synaptic tracing could be tested by co-culturing self-tracing neurons with primary rat neurons. This would serve as a proxy for lengthier transplantation of NSCs into an animal. Self-tracing NSCs underwent 2 weeks of neural induction prior to the addition of primary rat cortical neurons isolated from E17-19 pups. Co-culture was performed for an additional 2 weeks. Longer co-cultures were attempted but increased the risk of bacterial contamination and primary cell death. To differentiate between human and rat cells, STEM121 staining was used to reveal the distribution of human self-tracing neurons (Figure 13B). MAP2 staining further confirmed the presence of all neurons in culture, including rat primary neurons. MAP2 was paired with a quantum dot Q565 antibody to prevent emission spectrum overlap with other markers. However, Q565 excitation overlaps with DAPI and resulting in unusual nuclear staining. Nevertheless, WGA staining was clearly seen surrounding DAPI in $STEM121^-$ rat

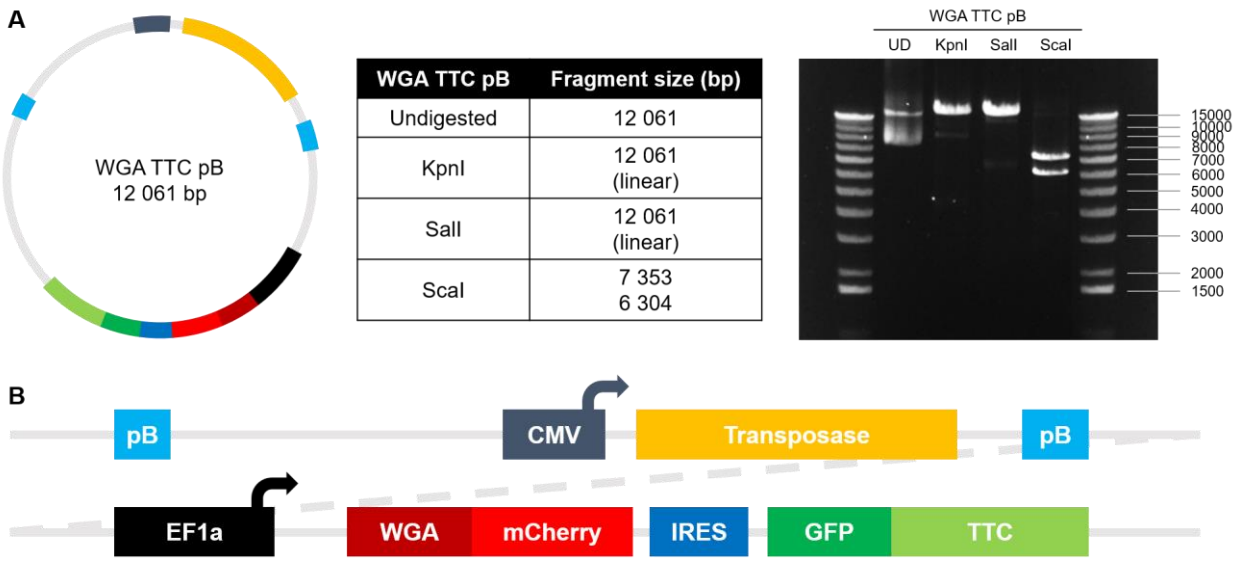
neurons (Figure 13C). Cytosolic TTC signal was also seen in a smaller population of rat neurons. Co-culture of wild-type NSCs, without any fluorescent tags, with rat primary neurons showed no WGA or TTC signal (Supplementary Figure 5). Together, these findings demonstrate that differentiate self-tracing NSCs so indeed integrate with rodent neurons and form synaptic connections.

3.4.2 Preliminary data from *in vivo* transplantation of self-tracing NSCs

Moving towards transplantation in a rodent model of SCI, RNU rats were injured with a 23 g contusion-compression model at the C6/7 level. 8 weeks post-SCI, self-tracing NSCs were prepared for transplantation at the epicentre and 4 additional sites 1 mm rostral and caudal to the lesion at a depth of 1 mm. Animals were kept for 12 weeks post-transplantation to allow for migration, differentiation, and integration of grafted cells into the host tissue. Firstly, long-term transgene expression of WGA-mCherry and GFP-TTC was examined (Figure 14). IHC demonstrated sufficient WGA and TTC signal when enhanced with antibody staining. Secondly, to see whether tracing occurred, we focused on finding cells that were only single-positive WGA^+ or TTC^+ cells. Distinct single-positive cells were identified suggesting that tracing occurred.

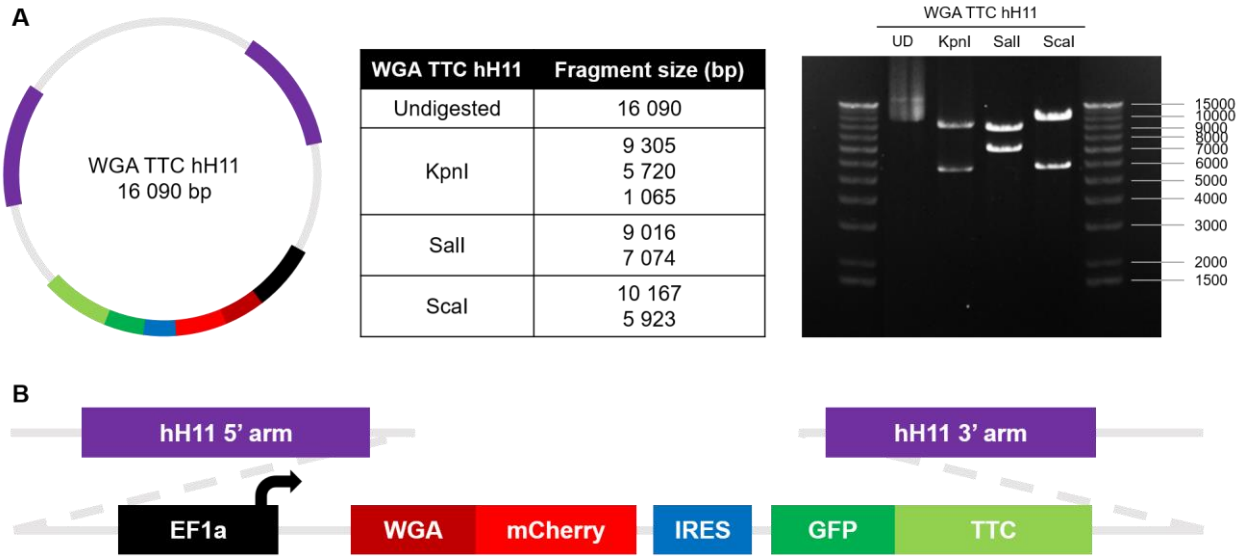
3.4.3 Conventional viral tracing shows limited tracing

As a brief qualitative evaluation of the effectiveness of conventional viral tracing techniques, naïve female Wistar rats were used for HSV-GFP and PRV152 tracing (n=3 per group). HSV tracing from the sensorimotor cortex was only detectable in the cortex surrounding the injection site and in very few neurons the ventroposterior nucleus of the thalamus (Figure 15). Screening of brainstem sections and spinal cord showed no traced cells. Similarly, PRV152 tracing of the flexor digitorum profundus, performed by M.R. at Drexel University, was only seen in a few cells in the dorsal and intermediate grey matter of the cervical spinal cord after DAB and cresyl violet staining.



WGA TTC pB	Fragment size (bp)
Undigested	12 061
KpnI	12 061 (linear)
SalI	12 061 (linear)
ScaI	7 353 6 304

Figure 5 Plasmid map of self-tracing NSC (pB). (A) Diagnostic restriction enzyme cuts were used to determine whether the final plasmid was correctly assembled. (B) Box-line diagram highlighting key components of DNA sequence, including constitutive promoters CMV and EF1a, transposase enzyme for genome integration, fusion protein tracers WGA-mCherry (anterograde), and GFP-TTC (retrograde). Abbreviations: CMV = cytomegalovirus; EF1a = elongation factor 1 alpha; GFP = green fluorescent protein; IRES = internal ribosomal entry site; pB = piggyBac; TTC = tetanus toxin fragment C; WGA = wheat germ agglutinin



WGA TTC hH11	Fragment size (bp)
Undigested	16 090
KpnI	9 305 5 720 1 065
SalI	9 016 7 074
ScaI	10 167 5 923

Figure 6 Plasmid map of self-tracing NSC (hH11). (A) Diagnostic restriction enzyme cuts were used to determine whether the final plasmid was correctly assembled. (B) Box-line diagram highlighting key components of DNA sequence, including constitutive promoter EF1a, homologous regions of the human H11 locus for sequence alignment and integration, fusion protein tracers WGA-mCherry (anterograde), and GFP-TTC (retrograde). A separate plasmid for Cas9 expression was used for host integration (not shown). Abbreviations: EF1a = elongation factor 1 alpha; GFP = green fluorescent protein; hH11 3' arm = human Hipp11 3' homologous arm; hH11 5' arm = human Hipp11 5' homologous arm; IRES = internal ribosomal entry site; TTC = tetanus toxin fragment C; WGA = wheat germ agglutinin

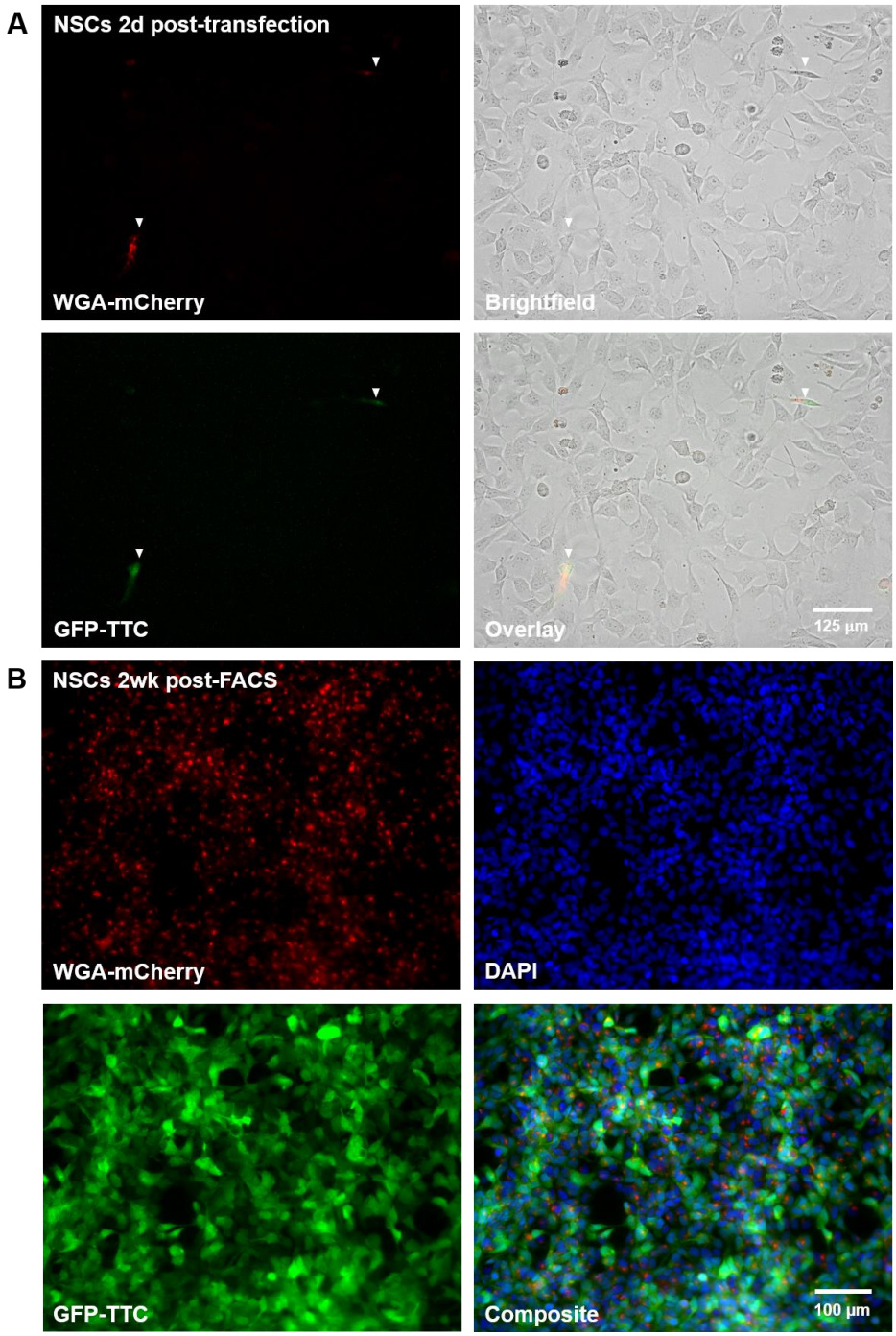


Figure 7 Monoclonal line of self-tracing NSCs. (A) Approximately 1% of NSCs demonstrated tracer expression 2 days post-transfection. (B) Ubiquitous expression of both WGA-mCherry and GFP-TTC were seen in NSCs 2 weeks post-FACS. Representative images are shown for WGA TTC (pB). Abbreviations: GFP = green fluorescent protein; pB = piggyBac; TTC = tetanus toxin fragment C; WGA = wheat germ agglutinin

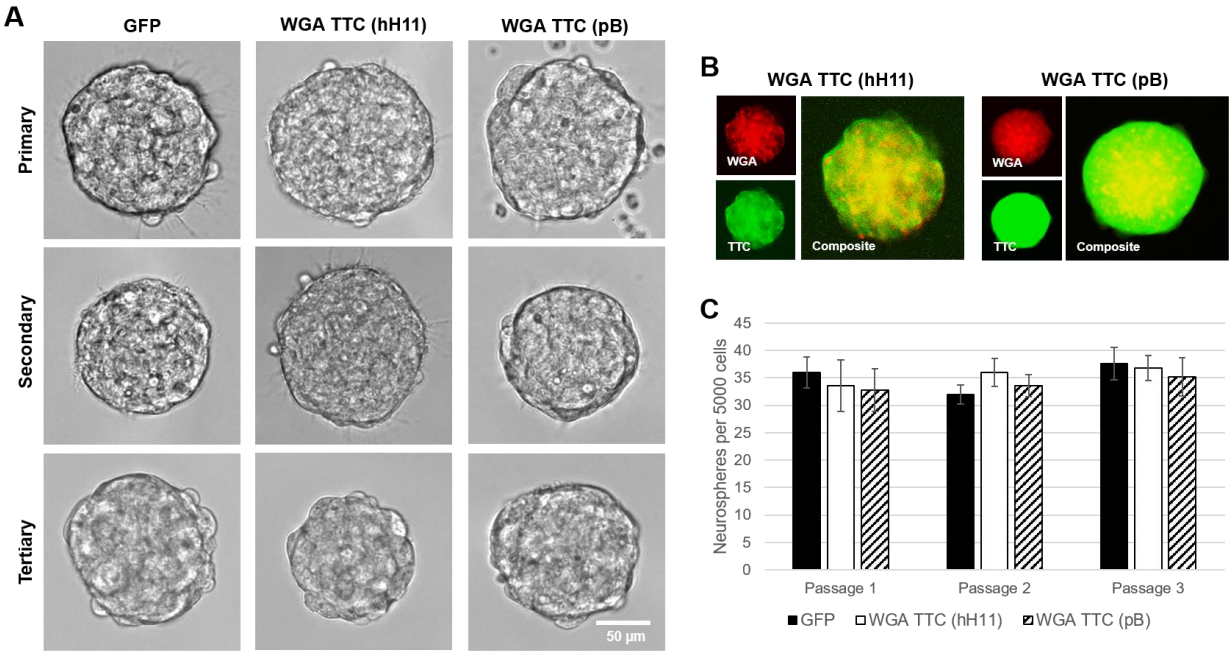


Figure 8 Self-tracing NSCs demonstrate typical proliferative properties. (A) GFP and self-tracing NSCs were cultured for 7 days to form primary neurospheres. Neurospheres were subsequently triturated and plated for an additional 7d days for form secondary and tertiary neurospheres. Representative brightfield images are shown for all cell lines. (B) Fluorescent images of both self-tracing lines of NSCs demonstrate ubiquitous expression of WGA and TTC. (C) After transferring and fixing neurospheres to a Geltrex-coated plate the number of neurospheres greater than 50 µm were quantified. Genetically modified NSCs did not show any difference in proliferative or self-renewal properties. Error bars display SEM, n=3 per group. One-way ANOVA corrected for multiple comparisons by Tukey's post hoc test, n.s. ($p > 0.05$). Abbreviations: GFP = green fluorescent protein; hH11 = human Hipp11 locus; pB = piggyBac; TTC = tetanus toxin fragment C; WGA = wheat germ agglutinin

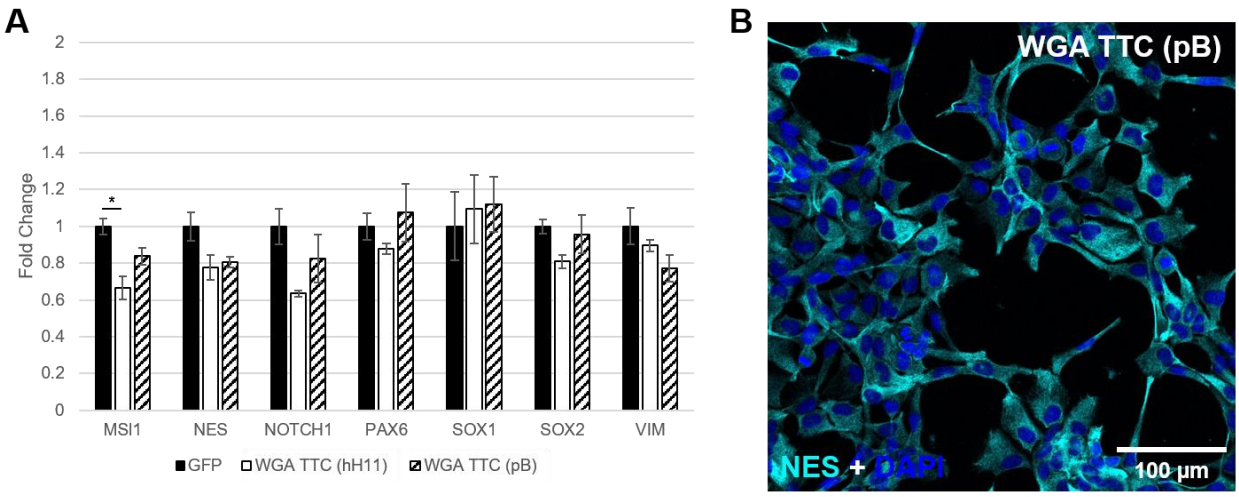


Figure 9 Self-tracing NSCs express typical NSC markers. (A) GFP and both lines of genetically engineered self-tracing NSCs demonstrate no difference in NSC marker expression by RT-PCR. All groups were compared to wild-type human iPSC-derived NSCs. Error bars display SEM, n=5 per group. One-way ANOVA corrected for multiple comparisons by Tukey's post hoc test, *$p < 0.05$. (B) Representative confocal image of self-tracing NSCs (pB) show expression of nestin. Abbreviations: hH11 = human Hipp11 locus; NES = nestin; TTC = tetanus toxin fragment C; WGA = wheat germ agglutinin

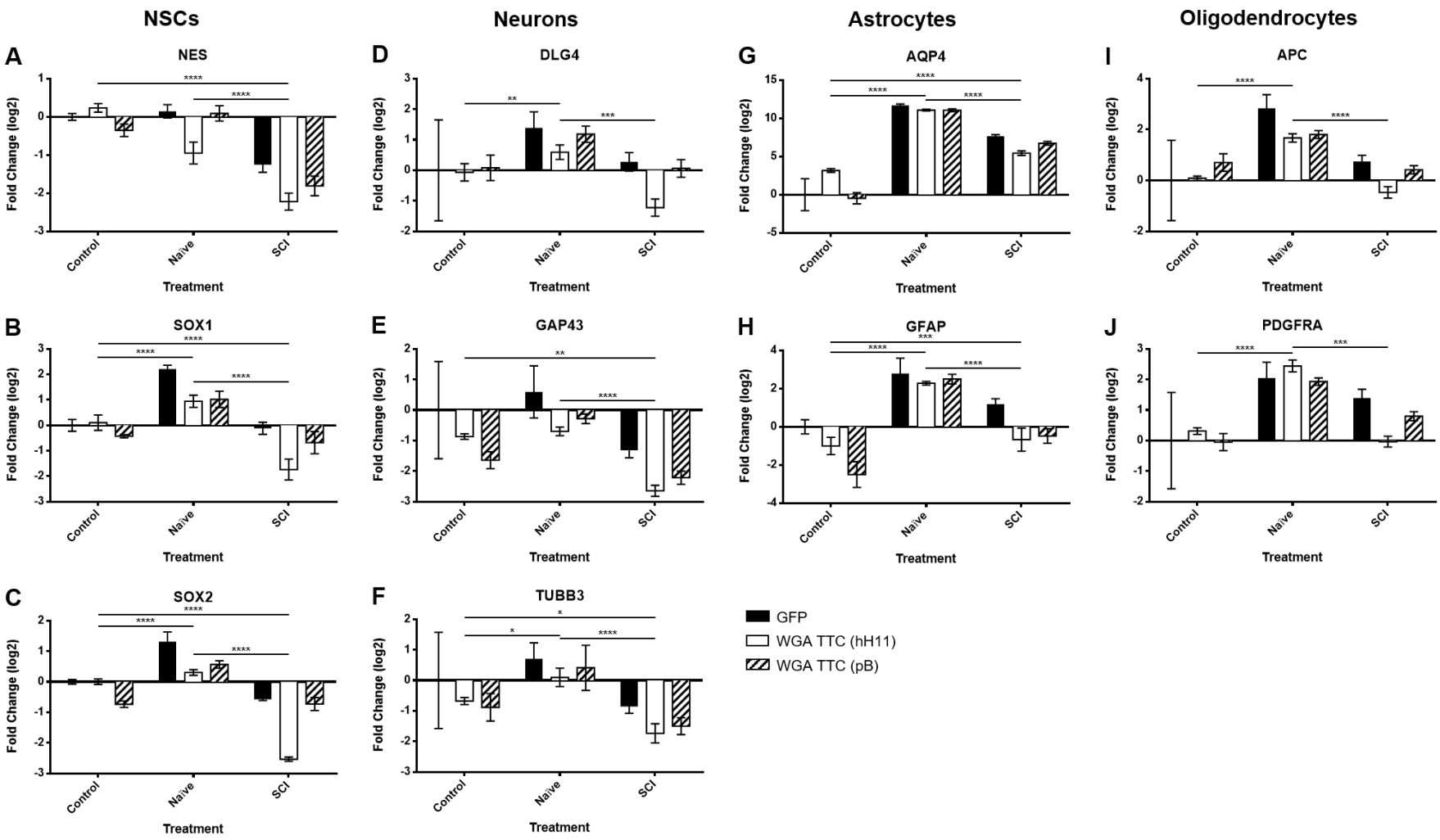


Figure 10 Differentiation profile of self-tracing NSCs by RT-PCR. GFP and self-tracing NSCs were treated with spinal cord homogenate from a naïve or 8 week-injured RNU rat for 1 week. NSCs cultured in SFM were used as a control. (A-C) NSC, (D-F) neuron, (G,H) astrocyte, and (I,J) oligodendrocyte markers were examined. Error bars display SEM, n=3 per group. Two-way ANOVA corrected for multiple comparisons by Tukey's post hoc test, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

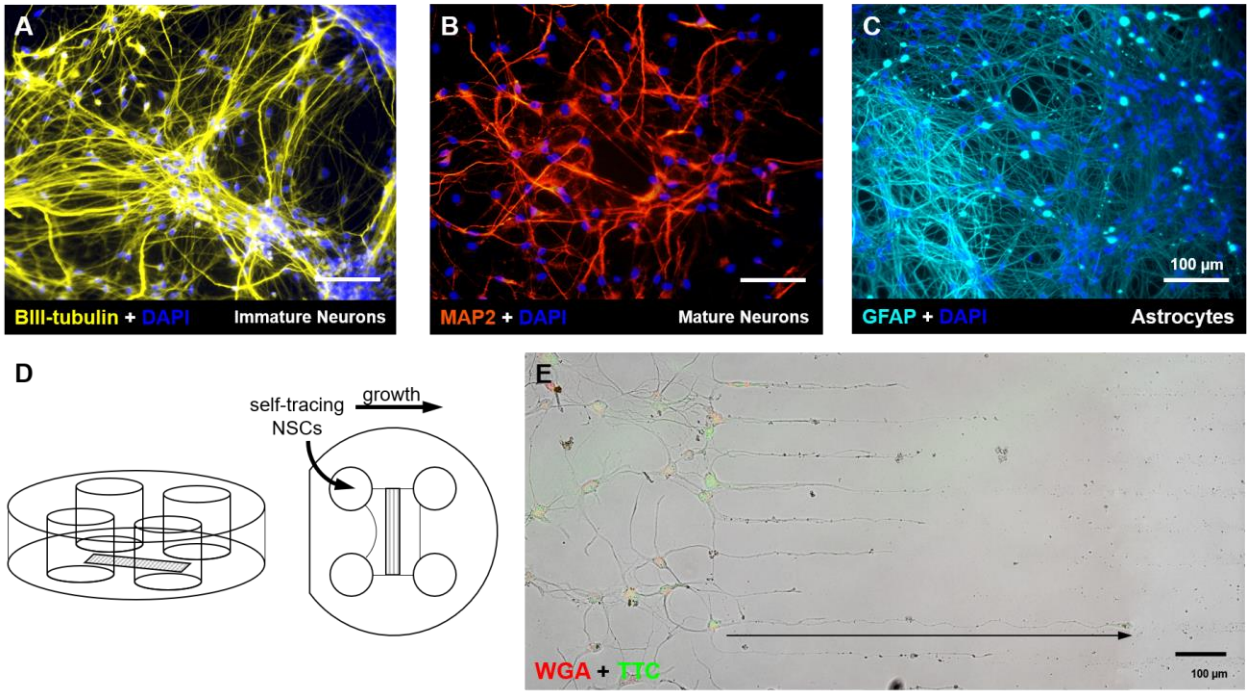


Figure 11 Self-tracing NSC can differentiate into neurons and astrocytes. (A,B) Self-tracing NSCs treated with neurobasal media with a cocktail of growth factors can differentiate into neurons. (A) Immature neurons were detectable within 1 week of induction and (B) more mature neurons were seen after 2 weeks. (C) Using 1% FBS, self-tracing NSCs became astrocytes within 1 week of induction. Representative images are shown of self-tracing (pB) cells. Images taken at 20X. (D) Diagram of Campenot chamber used to see directed axon growth. (E) NSCs differentiated to neurons over 2 weeks in a Campenot chambers were seen to grow long axons.

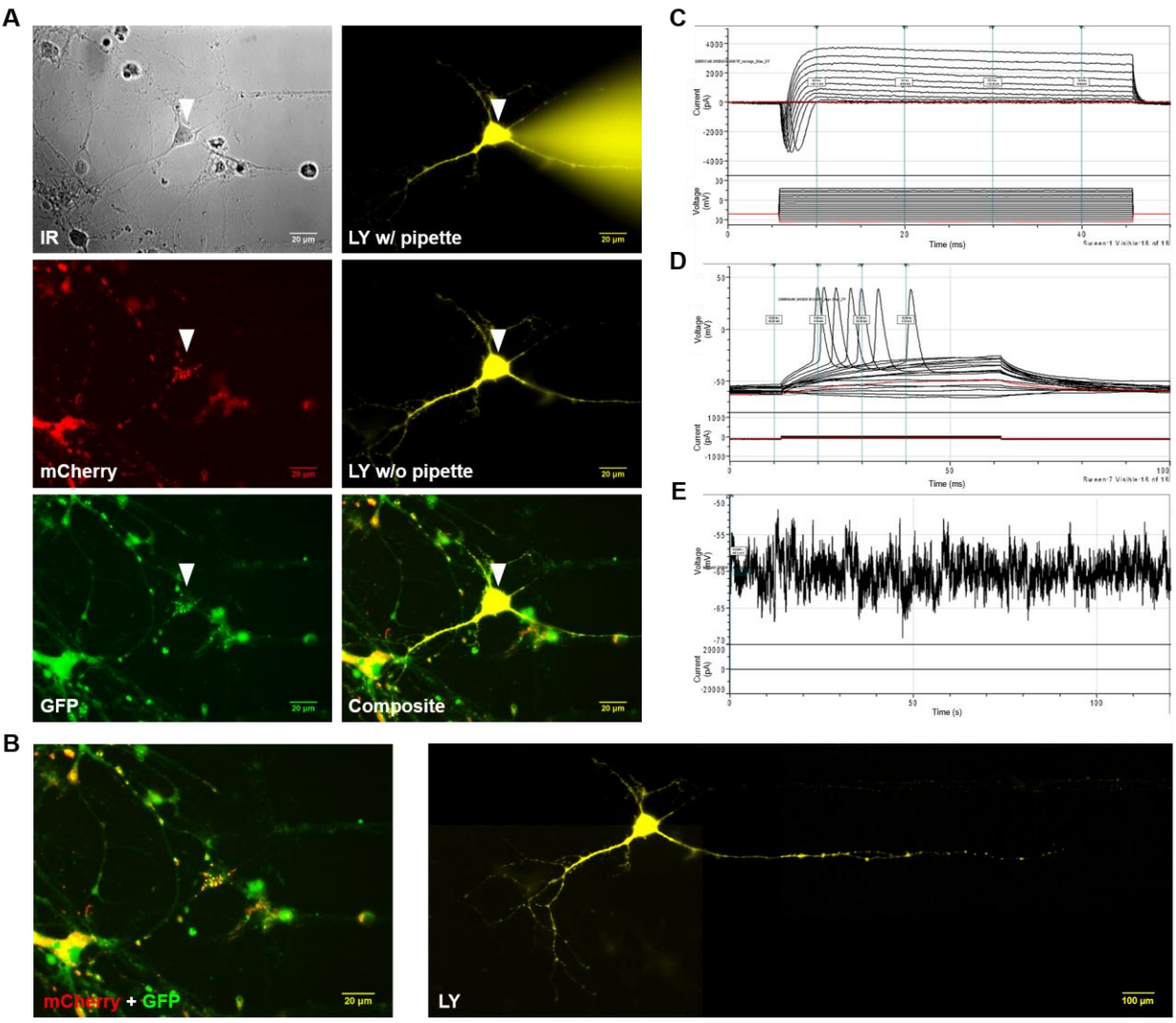


Figure 12 Self-tracing NSC-derived neurons are electrically functional. (A) Self-tracing NSCs were differentiated to neurons over 4 weeks. Cell were observed under IR and epifluorescent microscopy to identify healthy neurons for whole-cell recording. Arrowheads indicate patched cell. Once successful patching was achieved, neurons were injected with LY. Representative images of WGA-TTC (pB) NSCs are shown. (B) Composite image of mCherry and GFP expression confirm patched cells maintained expression of both molecular tracers, WGA-mCherry and GFP-TTC. Stitched image of LY-injected neuron shows complete cell morphology. (C) Voltage clamp reveal large inward Na+ current and large outward K+ current typical of neurons. (D) Current clamp demonstrated that differentiated neurons fire action potentials once membrane potential reached threshold. (E) 2 min recordings of current changes did not show any spontaneous synaptic activity. Abbreviations: LY = lucifer yellow

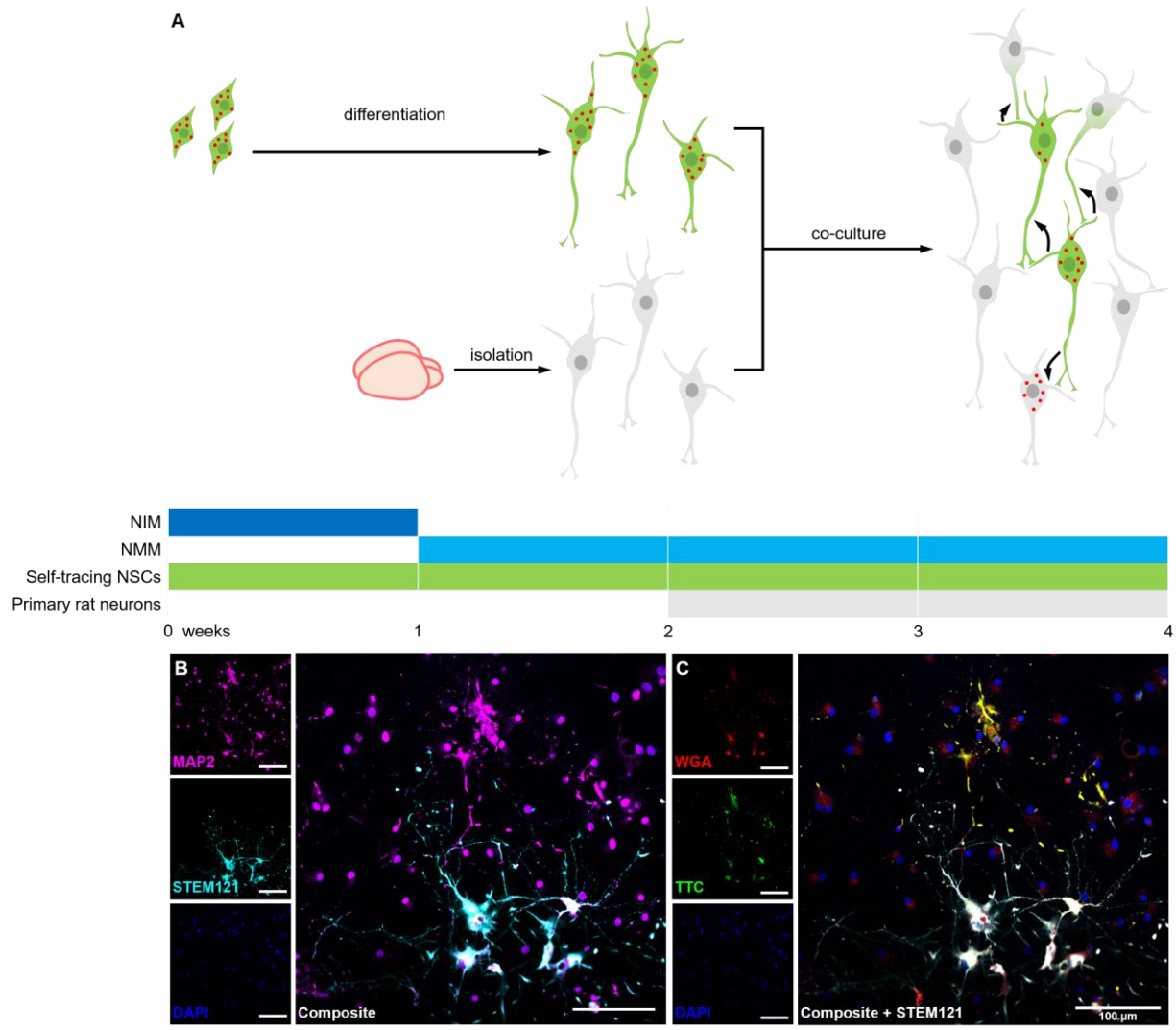


Figure 13 Self-tracing NSCs integrate and trace primary rat neurons *in vitro*. (A) Design of *in vitro* co-culture of self-tracing NSCs with primary rat cortical neurons. Self-tracing NSCs were plated and differentiated to neurons for 2 weeks prior to adding primary rat cortical neurons. Cells were left to mature and integrate over an additional 2 weeks. (B) Representative image of co-culture. STEM121 reveals distribution of human self-tracing NSC-derived neurons while MAP2 stains all neurons, including rat cells. (C) The same field of view shows dual positive WGA and TTC expression in human cells, but single WGA^+ or TTC^+ rat neurons. Abbreviations: MAP2 = microtubule-associated protein 2; TTC = tetanus toxin fragment C; WGA = wheat germ agglutinin

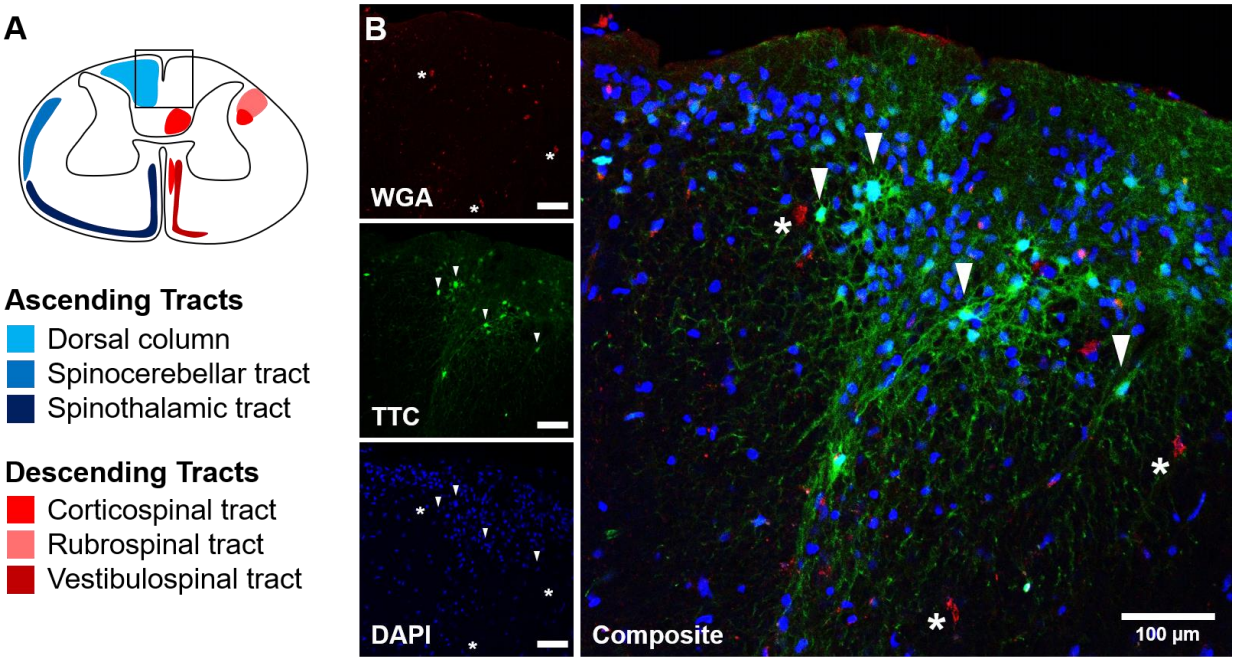


Figure 14 Self-tracing NSCs maintain tracer expression 12 weeks post-transplant. (A) Tract organization in a rodent cervical spinal cord for reference. The box marks the approximate region shown in the representative image. (B) Strong tracer expression is still observed 12 weeks post-transplantation. Asterisks mark WGA+/TTC- cells while arrowheads mark WGA-/TTC+ cells. Representative image of the dorsal funiculus at the epicentre of a 12 week-post self-tracing NSC transplant into an 8 week SCI (23 g, C6/7) RNU rat. Image taken at 20X.

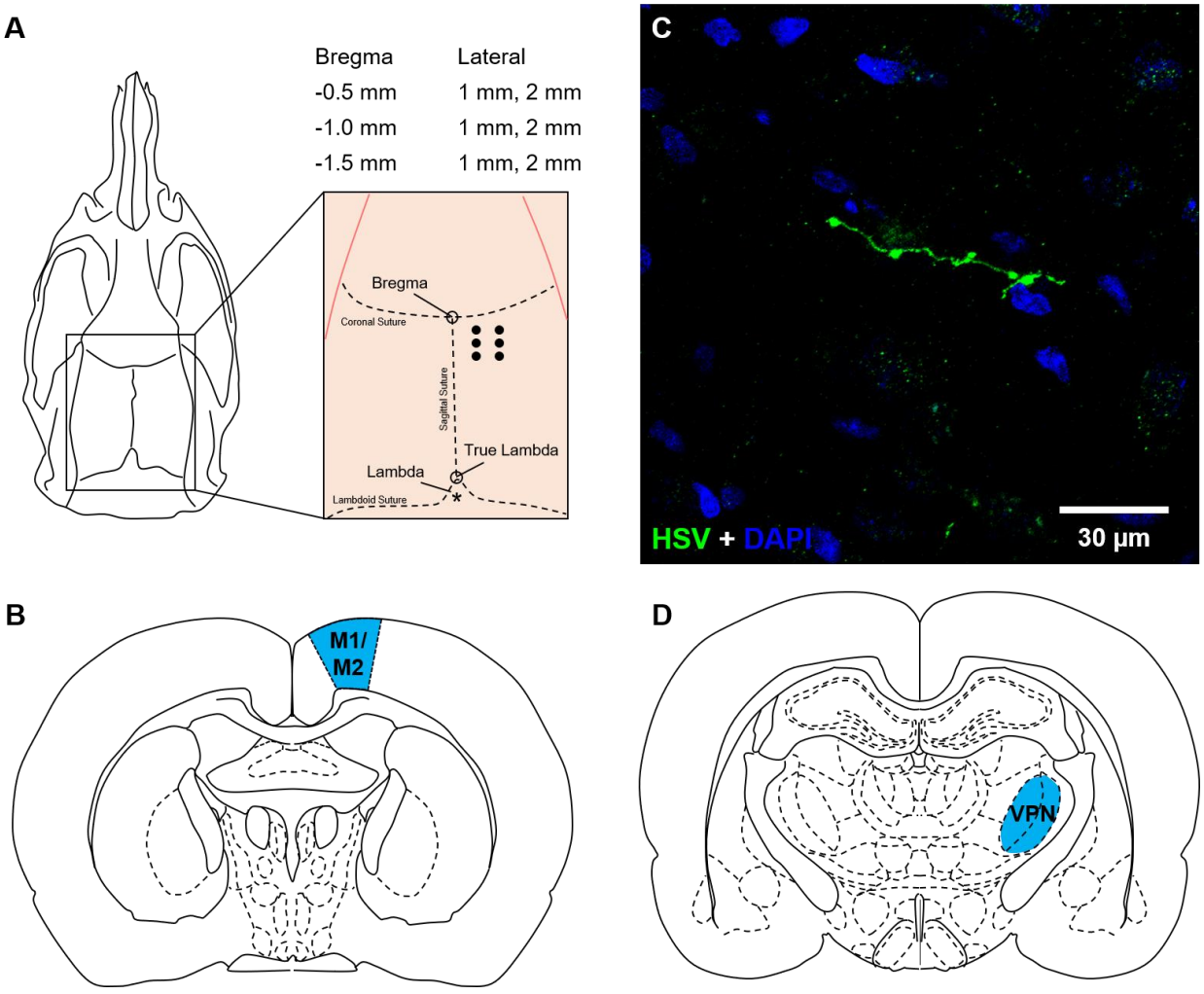


Figure 15 HSV injection resulted in sparse tracing of thalamic neurons. (A) Diagram showing landmarks and injection sites for surgery. (B) -1.0 mm Bregma section is shown. Highlighted is the corresponding M1/2 cortical region where injections were targeted. (C) Representative confocal image of traced neuron using maximum projection. Image taken at 63X. (D) Traced cells were observed within the ventroposterior nucleus of the thalamus in the right hemisphere. Abbreviations: HSV = herpes simplex virus; M1/2 = primary/secondary motor cortex; VPN = ventroposterior nucleus

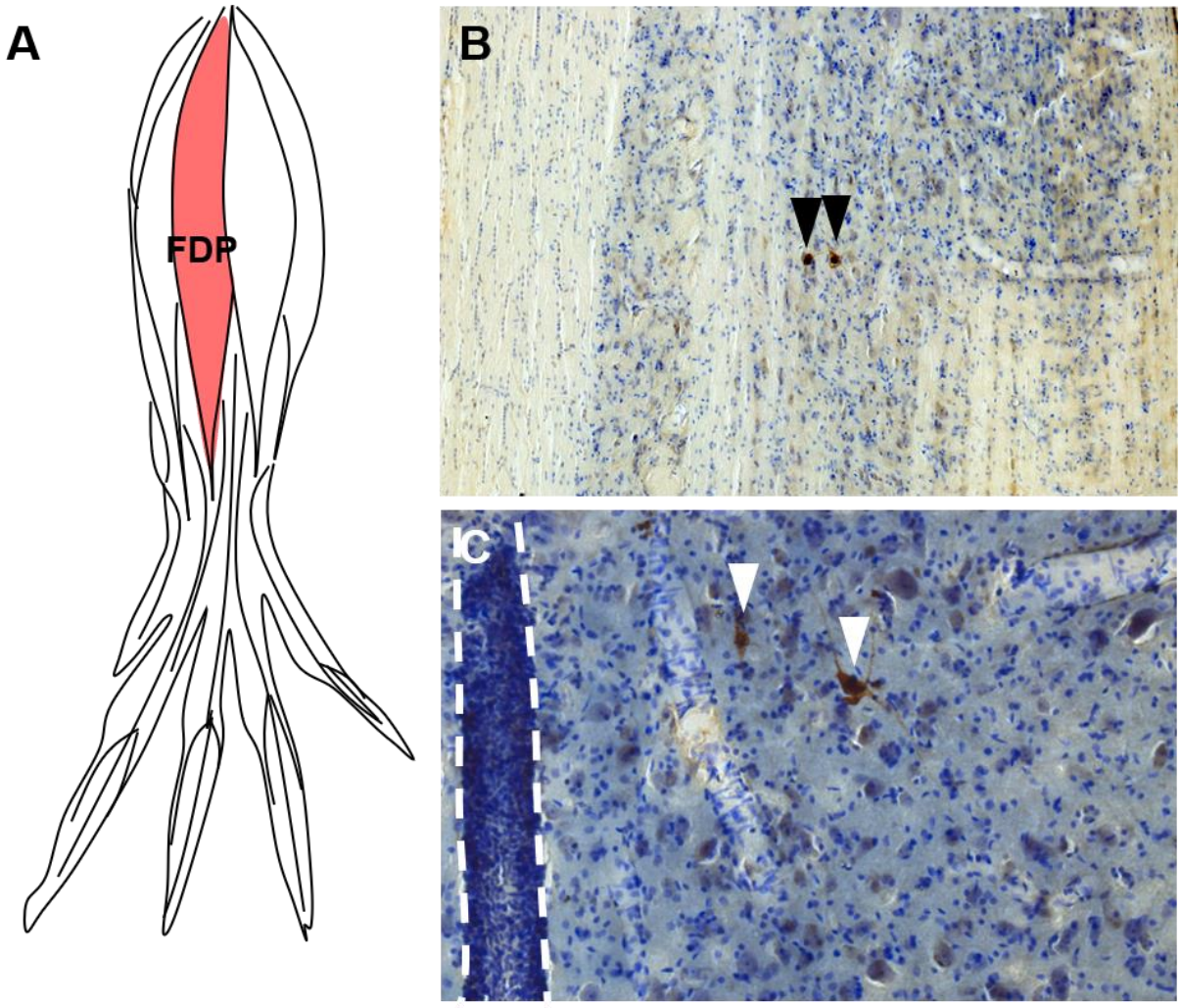


Figure 16 PRV injection resulted in sparse tracing of spinal interneurons. (A) Diagram depicting the approximate location of rat forearm muscle groups with the flexor digitorum profundus highlighted. (B) DAB staining shows few cells traced with PRV (brown) in the dorsal grey matter. Cresyl violet was used as a counterstain. (C) Some interneurons are also traced in the intermediate grey matter. Central canal marked by dashed line. Images were courtesy of Margo Randelman & Dr. Michael Lane, Drexel University. Abbreviations: FDP = flexor digitorum profundus; PRV = pseudorabies virus

Chapter 4
Discussion

4 Discussion

4.1 Summary of Results

Through our work, we have demonstrated how hiPSC-NSCs can be genetically engineered to constitutively express WGA-mCherry and TTC-GFP without modifying inherent NSC characteristics. This was achieved using both a non-viral piggyBac transposon system and CRISPR-Cas9 system. Both methods produced reliable transgene expression post-FACS and expansion, with the self-tracing (pB) line demonstrating greater expression. Self-renewal and proliferative potential were retained as shown by the successful generation of primary, secondary, and tertiary neurospheres. Self-tracing NSCs did not show abnormal proliferation, which would indicate greater tumorigenic risk, when neurospheres were quantified. Further confirmation of normal NSC phenotypes was achieved by RT-PCR and ICC. Self-tracing NSCs appeared to demonstrate multipotency when treated with spinal cord homogenate, and *in vitro* differentiation of NSCs could generate electrically functional neurons. Importantly, these self-tracing neurons demonstrated successful of tracing of primary rat neurons *in vitro*, and possible *in vivo* as well.

4.2 Discussion

4.2.1 From remyelination to neuronal relay

If SCI results in the loss of neurons, astrocytes, and oligodendrocytes, a logical treatment approach would be to replace the lost and damaged cells. In this line of thought, NSCs are an obvious candidate for cell-based therapies in the context of SCI. Developments in stem cell research have also allowed NSCs to be generated from various sources, both safely and effectively, making NSCs an even more attractive approach to treating SCI. Specifically, our lab's translation-focused mindset led us to use human iPSC-derived NSCs.

To put theory to test, NSC transplantations in multiple preclinical SCI models, rodent and non-human primates included, have generally conferred some improvements in neurologic recovery (Bonner and Steward, 2015; Mothe et al., 2008; Tetzlaff et al., 2011). Over the years, researchers can summarize the main mechanisms by which NSCs promote functional improvements as

follows: neuroprotection (Hawryluk et al., 2012; Lu et al., 2003), immunomodulation (Cusimano et al., 2012), angiogenesis (Kobayashi et al., 2012; Li et al., 2014), remyelination (Eftekharpour et al., 2007; Karimi-Abdolrezaee, 2006; Plemel et al., 2011), and neuroplasticity (Kadoya et al., 2016; Lu et al., 2012). Previously, our lab placed a greater emphasis on NSC-to-oligodendrocyte differentiation to promote remyelination, in part, due to the natural tendency of our NSC line to grow into a neuroglial fate (Eftekharpour et al., 2007; Karimi-Abdolrezaee, 2006). We capitalized on this finding by generating a line of NSCs which were biased more towards oligodendrocyte differentiation, while still preserving their multipotency (Nagoshi et al., 2018). Compared to control NSCs, oligodendrogenic NSCs were associated with greater white matter sparing, BBB score, stride length, and swing speed. Results of this study, along with literature on OPC-based therapies, further supported the significance of remyelination in SCI recovery.

However, other researchers have downplayed the importance of oligodendrocytes in locomotor recovery. A study by Duncan et al. demonstrated that PDGFRa^{+} OPCs with an inducible myelin regulatory factor-knockout resulted in a reduction of myelination in transgenic mice (2018). Surprisingly, the decrease in remyelination did not affect spontaneous recovery following SCI. This prompted us to re-evaluate the differences and potential advantages of NSC over OPC use: multipotent NSCs can also differentiate into astrocytes and neurons.

NSC-to-astrocyte differentiation is an interesting concept given that most astrocytes in the SCI microenvironment become reactive and contribute to the formation of the glial scar (Herrmann et al., 2008). Ironically, preventing scar formation or ablation of astrocytic scars does not lead to axon regeneration (Anderson et al., 2016). Rather than tackling this complex mechanism, we sought to better understand NSC-to-neuron differentiation.

Lu et al. have presented remarkable long-distance growth of graft-derived axons in a T3 complete transection SCI model which was associated with significant BBB recovery and evoked potential latency recovery (2012). Although the authors inferred synaptic integration of grafted cells by dual-positive GFP and synaptophysin staining, true synaptic connections could not be verified without tracing. A subsequent study demonstrating similar neural differentiation and engraftment focused on uncovering the neuroplasticity of endogenous CST regeneration (Kadoya et al., 2016). While the CST fibres appeared to regenerate caudal to the lesion, improvements in forelimb function, assessed by the food-pellet reaching task, were not

significant. Another study from the same group then examined the specific neural subtype fate of NSC grafts. NSCs were found to organize into dorsal horn-like domains and were innervated specifically by host sensory, but not CST neurons (Dulin et al., 2018). Unfortunately, no sensory test data was provided.

Nevertheless, robust neural differentiation and apparent synapse formation support neuronal relay as a plausible mechanism underlying NSC-mediated recovery. In order to definitively answer whether synaptic integration occurs between grafted neurons and host cells, trans-synaptic tracing is necessary.

4.2.2 Development of self-tracing vector

Tract tracing is not a novel concept in stem cell and SCI research. Numerous publications have made use of viral and non-viral tracers, mainly to study neuroplasticity of host neurons. As simple as tracing protocols may appear on paper, in practice, they are plagued with challenges. One of which is the detection of tracers within the spinal cord following injection originating at meaningful distal sites (i.e. functional cortical regions, muscle groups, etc.) as exemplified in Figures 15,16. While insufficient optimization may be to blame for our poor results, we believe it is important to have a robust tracing method that guarantees tracing. This holds true especially when considering the need to detect trans-synaptic tracers within both grafted cells and their up- or downstream connections.

Rather than randomly targeting a small population of graft neurons, we conceived having tracing originate from transplanted NSCs. Doerr et al. proposed a very similar idea, using a transgenic mRFP-expressing neuroepithelial stem cell which was also transduced with a rabies virus-inducible GFP tracing vector (2017). Introduction of a modified rabies virus permitted viral replication and monosynaptic retrograde tracing. These transgenic cells were undoubtedly successful in mapping transplant innervation after grafting into a mouse brain. Still, for our purposes, we found several limitations with this study. Firstly, we would like to see both anterograde and retrograde trans-synaptic tracing. Secondly, no characterization of the neuroepithelial stem cells post-genetic modification was done. Lastly, while this was an inducible system, in an SCI and transplantation experiment, additional intrathecal injections may cause further unintentional injury.

For these reasons, we aimed to develop our own version of self-tracing NSCs which could constitutively express both anterograde and retrograde trans-synaptic tracers.

4.2.3 Bioengineering strategies

Avoiding the potential neurotoxic effects of viral tracing methods, we preferred to use WGA and TTC as trans-synaptic molecular tracers. We designed a fusion WGA-mCherry sequence, analogous to the approach used to conjugate WGA to other fluorophores (Goshgarian and Buttry, 2014). The GFP-TTC sequence was based on the original paper by Kissa et al. (2002). The internal ribosomal entry site (IRES) allowed both tracer expressions to be driven by a single EF1a promoter, which has been shown to maintain high expression of transgenes across multiple cell types, including ESCs (Norrman et al., 2010; Qin et al., 2010). The woodchuck hepatitis virus post-transcription regulatory element (WPRE) was added downstream to further enhance expression (Klein et al., 2006). The first iteration of the self-tracing vector was packaged within a pB backbone given prior success with stable genetic modification using this system (Woltjen et al., 2009). An alternative CRISPR-Cas9 dependent self-tracing vector was targeted to hH11 region, which has been reported as a safe-harbour locus (Ruan et al., 2015).

Although AAV has been repeatedly shown to be a reliable site for CRISPR/Cas9-mediated DNA editing via homology-directed repair in multiple systems (Chew et al., 2016; Dai et al., 2019; Ge et al., 2016; Nishiyama et al., 2017; Ohmori et al., 2017; Senís et al., 2014; Swiech et al., 2015), we made a conscious decision to use the H11 locus. This allows our lab to combine multiple vectors, each with unique host integration sites, in the future, opening the door to future combinatorial treatments of trophic factors (Jiao et al., 2017; Liu et al., 2017; Robinson and Lu, 2017; Rosich et al., 2017; Zhang et al., 2018) and scar-degrading enzymes (Alluin et al., 2014; Karimi-Abdolrezaee et al., 2012; Nori et al., 2018; Suzuki et al., 2017) packaged within a single NSC line.

Expression of tracer molecules, as interpreted by mCherry and GFP detection, appeared robust, suggesting the careful selection of DNA elements and sequence design was successful. While fluorescence should correspond to tracer expression, direct assessment or quantification of WGA and TTC expression was not quantified. This could have been done by western blot to examine WGA and TTC bands which should appear at the combined molecular weight of the tracers and fluorescent proteins. This is unlikely to pose a major concern given that tracing of rat primary

cortical neurons in co-culture was observed (Figure 13), but not when neurons were plated with wild-type NSCs (Supplementary Figure 5).

Again, the pB transposon system was used because of its relative ease of use and ubiquitous transgene expression. However, the high expression may be due to multiple insertion sites given that the pB transposase recognizes inverted terminal repeats (ITRs) flanking the vector and inserts the complete sequence into any TTAA chromosomal site (Li et al., 2013; Zhao et al., 2016). PCR and/or fluorescent *in situ* hybridization can be used to confirm the number of insertion sites. Another point is that the transposon system has the potential to cause large chromosomal translocation. Karyotyping the self-tracing NSCs would provide more assurance that these NSCs have not been unintentionally mutated. We believe this is unlikely due to our characterization of various NSC phenotypes.

Supplementary Figure 4 also shows that self-tracing (hH11) NSCs loss GFP intensity over multiple passages. Ruan et al. previously claimed that the pig H11 locus was an efficient site for CRISPR/Cas9-mediated knock-in of GFP in fetal porcine fibroblasts, embryos, and eventually piglets born 114 days after embryo activation (2015). In our study, we did not assess GFP-TTC expression in cells for nearly as long-term, but we already started with decreased expression of GFP compared to mCherry, which was detectable by eye. This was expected as previous work studying the relative expression of transgenes upstream and downstream of IRES concluded that IRES-depending second gene expression generally ranges from 20 to 50% of the promoter-driven first gene (Mizuguchi et al., 2000). We chose to place GFP-TTC as the second gene in the hopes that a cytosolic localization (Coen et al., 1997) would be easier to detect than punctate WGA signal (Ruda and Coulter, 1982). In our pB line, we also compensated for lower expression by adding a WPRE sequence. Unfortunately, no single- or dual-cutting RE sites were available to retain WPRE in the hH11 self-tracing NSCs for Gibson subcloning. Another explanation is that that silencing of transgene expression by the host genome occurred by either DNA methylation or post-transcriptional RNA degradation (Matzke et al., 2000). In the future, this can be addressed by adding a ubiquitous chromatin opening element (UCOE), which has proven to be effective in primary haematopoietic cells and pluripotent stem cells (Pfaff et al., 2013).

4.2.4 Self-tracing NSCs retain normal NSC phenotypes

One of our criticisms of previous rabies virus-depending tracing cells was the lack of cell characterization. We demonstrated the safety of using pB transposon and CRISPR/Cas9 systems to genetically modify NSCs while preserving NSC properties. Our self-tracing NSCs maintained normal self-renewal and proliferative properties and expressed hallmark NSC markers at similar levels to control NSCs.

NES and NOTCH1 also slightly downregulated in self-tracing NSC. NES encodes nestin, a cytoskeletal intermediate filament protein normally expressed in NSCs (Zhang and Jiao, 2015). Knock-down of NES expression in NSCs was found to decrease cell migration and contractility (Yan et al., 2016). NES has also been implicated in NSC self-renewal, proliferation, and survival (Bernal and Arranz, 2018). On the other hand, tight regulation of Notch-1 signalling is important in maintaining NSC quiescence (Zelentsova et al., 2017). Notch-1 also acts as a switch for NSC differentiation to either glial cells or neurons, with greater expression promoting more astrocyte differentiation (Zhou et al., 2010). While neither marker was significantly downregulated, we should be mindful in assessing migration and astrocyte vs. neuron differentiation when assessing *in vivo* transplantation of self-tracing NSCs in the future.

Expression of MSI1, encoding RNA-binding protein musashi 1, was the only marker to be differentially expressed in control and self-tracing NSCs, with decreased expression in self-tracing hH11 cells. Musashi plays an essential role in asymmetric cell division of *Drosophila* proliferating neuroblasts by regulating the translation of target mRNA (Okano et al., 2005). Mammalian homologue MSI1 is functionally conserved, as its high expression in both fetal and adult NSCs is crucial for NSC self-renewal. Although quantification of the neurosphere assay did not reveal any difference in the number of neurospheres generated per 5000 cells, it is possible that the average size of neurospheres may have differed slightly, but image analysis would be needed to support this claim.

4.2.5 Self-tracing NSCs differentiate to neuroglial lineage

Self-tracing NSCs tended to express greater astrocyte and oligodendrocyte markers following spinal cord homogenate treatment, in both naïve and injured conditions. RT-PCR was used as a substitute for ICC given our limited quantity of tissue homogenate to treat multiple NSC lines

and replicates. While not the perfect experiment, this is a useful first step in still probing the differentiation profile of these NSCs.

Since we did not see convincing increases in neuron markers after inducing differentiation by homogenate treatment, we confirmed the neural potential of self-tracing NSCs by following a neural induction protocol. Not only did this result in the expression of cytoskeletal proteins TUBB3 and MAP2 over the course of 2 weeks, but these cells were also mature enough by 4 weeks post-neural induction to express active Na^+ and K^+ channels necessary for action potential firing. The lack of spontaneous synaptic activity recorded over 2 minutes warrants further discussion. It is important to note that the initial membrane potentials of our recorded cells typically ranged from -20 to -30 mV. We artificially held the membrane potential at -70mV, the typical resting membrane potential of neurons. This suggests that our neurons may not have been fully differentiated.

Xie et al. compared different iPSC to neuron differentiation protocols and concluded that direct differentiation of iPSC to neurons, without expanding NSCs, produced neurons that fired action potentials within 4 to 5 days, and repetitive action potentials within 35 to 38 days (2018). The protocols also required plating over an astroglial feeder layer to promote synapse formation. We were unable to follow this protocol given that we began this project using a previously generated iPSC-derived NSC line that has been stored in liquid N_2. Difficulties in maintaining an astrocyte feeder layer beneath a Campenot chamber also prevented us from optimizing neural maturation.

Revisiting previous NSC transplantation experiments published by our lab, our prior findings support our current results, with most NSCs tending to differentiate towards an astrocyte or oligodendrocyte phenotype (Eftekharpour et al., 2007; Karimi-Abdolrezaee, 2006; Karimi-Abdolrezaee et al., 2012; Nagoshi et al., 2018; Nori et al., 2018; Salewski et al., 2015; Suzuki et al., 2017; Zweckberger et al., 2016). It is important to note, however, that the relative abundance of neurons, astrocytes, oligodendrocytes, and undifferentiated NSCs varied between different cell lines. For our purposes, it would have been better to use a different NSC line, on that is more inclined to differentiate into neurons such as the ones seen in Yan et al.'s study (2007), the application of tracing neural relays post-transplantation. The beauty of our concept though lies in our self-tracing vectors themselves, which can be introduced into any NSC cell line of varying derivations.

4.2.6 Self-tracing NSCs do trace synaptic connections bidirectionally

To answer our initial hypothesis, we were successful in demonstrating that a genetically engineered NSC can trace its own synaptic connections. Based on our co-culture experiment between human self-tracing NSC-derived neurons and primary rat cortical neurons, both single-positive WGA and TTC neurons were observed without the presence of STEM121. Our negative control, using wild-type NSCs with no inherent expression of fluorescent proteins, confirmed that these findings were neither a result of background signal nor non-specific antibody binding.

Our findings reaffirm the use of conjugated WGA and TTC as trans-synaptic molecular tracers. WGA localized to small vesicles (Levy et al., 2015) within the soma and diffuse GFP-TTC (Coen et al., 1997; Kissa et al., 2002) were found throughout of traced rodent cells as expected. Interestingly the morphology of the human neuron in both Figures 12 and 13 resembles that of pyramidal neurons, with apical and basal dendrites, and a triangular cell body (Bekkers, 2011). Pyramidal neurons are known for the long axon projections, and these neuron subtypes comprise the upper motor neurons of major motor pathways, including the CST. It would be interesting to see whether these neurons derived from self-tracing NSCs indeed migrate and develop pyramidal-like morphology after transplantation in the spinal cord.

4.2.7 Self-tracing NSCs are found in the dorsal funiculus

12 weeks post-NSC transplant, GFP^+ and $mCherry^+$ cells were found in the dorsomedial aspect of the dorsal funiculus. Arrows and asterisks in Figure 14 indicate some single-positive cells, which should correspond to traced. However, this is a statement is made with extreme caution given that no co-labelling of STEM121 or neural markers were used to distinguish between grafted cells and host, nor neurons and glial cells. Morphology alone is inconclusive as it is very difficult to distinguish between the projections emanating from cell bodies. A deeper analysis and commentary of this limitation will be continued below.

From this initial experiment, what we can examine is the distribution of grafted cells within the spinal cord. Most cells are found within the dorsal white matter, close to the dorsal surface adjacent to midline. In comparison to the rat atlas, this region is consistent with the location of the DCML. Further investigation looking at longitudinal sections would help determine whether DCML neurons participated in neural relay through the graft. WGA and TTC signal did not appear as strong in the ventral aspect of the dorsal funiculus, where the fibres of the CST run.

Higher magnification images may be able to detect the presence of weaker tracer signal. Despite these limitations, the distribution of grafted cells prompts further investigation into the regeneration and/or relay of DCML and CST fibres.

4.3 Limitations

NSCs that constitutively express GFP and mCherry naturally raise technical considerations. The main challenge is the restricted use of marker co-labelling during ICC and IHC to define cell phenotypes and connectivity more precisely. For example, to confirm that self-tracing NSCs differentiate to neurons and form synaptic connections, three separate markers (i.e. STEM121, MAP2, synaptophysin) should be used. Normally this would be possible with wild-type NSCs. Even after expanding the number of stains possible by utilizing quantum dot antibodies, each with an identical 405 nm excitation peak but distinct emission spectra (Francis et al., 2017), there is still too much signal crossover for extensive visual characterization of self-tracing NSCs.

Largely an *in vitro* proof-of-concept study, we remain cautiously optimistic in translating these results *in vivo*. Our co-culture experiments used to test tracing involved specific conditions to promote neuron-only growth. *In vivo*, it is much more likely to see astrocyte and oligodendrocyte differentiation, as we expected from prior studies and our RT-PCR profile. It is possible to treat NSCs with spinal cord homogenate prior to co-culture and tracing, but the time-course of NSC differentiation has not yet been defined by this method.

The least developed experiment was clearly the *in vivo* proof-of-concept. Additional MAP2 and STEM121 staining would greatly benefit the study to affirm whether WGA+ or TTC+ were indeed neurons and whether the tracers were observed within the grafted human or host rat neurons. A separate evaluation of tracer uptake into host astrocytes and oligodendrocytes could also provide some indication as to how specific this tracing technique is. On top of this, while we did observe HSV and PRV tracing in naïve animals, it would be important to perform tracing in NSC transplanted animals to validate which method is more effective and efficient in tracing grafted neural relays.

Chapter 5
Conclusion

5 Conclusion

5.1 Novelty and impact

NSCs to treat SCI have been brought to clinical trials, but the significant neurological recovery seen pre-clinically has not been reflected as strongly in patient populations (Sahni and Kessler, 2010). One key gap in the development of stem cell therapies is understanding which motor and sensory pathways mediate recovery (Dedeepiya et al., 2014). But before this can be done, better tools are needed to trace trans-synaptic integration of grafts into host circuitry. Our study has addressed this gap by creating NSCs that can map out its synaptic connections after transplantation. This self-tracing vector has the potential to be inserted in other NSC lines to map out neural circuitry of both the brain and spinal cord.

5.2 Future directions

5.2.1 Inducible tracing system

While we purposely designed the self-tracing NSCs to constitutively express both trans-synaptic tracers we have also considered a variant of the plasmid with a controlled “on” switch. Based on a Cre-lox paradigm, inverted tracer sequences can be flanked by alternating pairs of inverted lox sites (Schnütgen et al., 2003). Application of Cre recombinase would cause the tracer sequence to first flip, and a secondary excision of now parallel lox sites (flip-excision, FLEx). See Figure 17 for the plasmid design. Consequently, a timed introduction of Cre recombinase can turn on tracer expression when needed. Refining control even more, a tetracycline or doxycycline-inducible system (tetracycline-On, Tet-ON) may be employed (Gossen et al., 1995; Gossen and Bujard, 1992; Orth et al., 2000). Transfection of either vector into NSCs can be performed and a similar experimental design can be followed to characterized stemness of the transgenic cells.

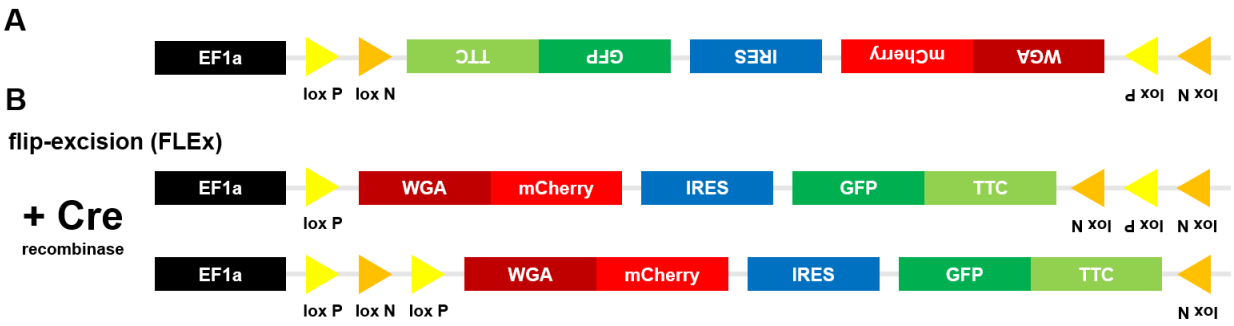


Figure 17 Inducible self-tracing plasmid using FLEx. (A) Box-line diagram highlighting key components of DNA sequence, including pairs of inverted lox P and lox N sites flanking an inverted sequence containing genes encoding trans-synaptic tracers (B) Possible flip-excision products allowing for induced tracer expression. Abbreviations: EF1a = elongation factor 1 alpha; GFP = green fluorescent protein; IRES = internal ribosomal entry site; TTC = tetanus toxin fragment C; WGA = wheat germ agglutinin

5.2.2 Promoting neural differentiation

We have briefly mentioned that our current NSC line may not be ideal to demonstrate *in vivo* tracing due to a low proportion of NSCs differentiating into neurons. This can be overcome by two means: use of a different line of NSCs; or promotion of neural differentiation by growth factors.

One school of thought among stem cell researchers is the inherent identity of NSCs dictate their behaviour in terms of proliferation, migration, and differentiation patterns (Nakafuku et al., 2008). Not only does this apply to differences in the developmental timeline at which NSCs are obtained (Qian et al., 2000), but some groups also believe that region-specific patterning of cells, caused by differential morphogen exposure during development (Bixby et al., 2002), can all contribute to variations in NSC phenotypes. With this in mind, it is likely that NSC lines generated from ESCs, iPSCs, or directly reprogrammed somatic cells, will all vary in neural differentiation potential. So far, our lab has tested genetically modified NSCs against our internal NSC controls. In the future, it is advisable to test our NSCs against other external sources of NSCs. We would predict that we would see high variability in neural differentiation efficiency, analogous to the variability seen by researchers examining different ESC lines for NSC generation (Yin et al., 2012).

Work from the Tuszynski Lab best exemplifies NSC to neuron differentiation by supplementing NSC transplants with trophic factors (Dulin et al., 2018; Kadoya et al., 2016; Lu et al., 2017, 2012, 2003). They achieve long-axon growth by combining NSCs with a cocktail of growth factors embedded within a fibrin matrix: BDNF, NT3, GDNF, IGF, bFGF, EGF, PDGF, aFGF, and HGF (Lu et al., 2014). FGF, EGF, and HGF are growth factors needed to sustain NSC survival, self-renewal, and proliferation (Arsenijevic et al., 2001), and is regularly added to culture media, as seen in Supplementary Table 2. IGF plays a similar role in self-renewal, but also promotes neurogenesis (Ziegler et al., 2015). More commonly associated with axon regeneration and sprouting, BDNF and GDNF further contribute to cell survival and neural differentiation (Chen et al., 2012; Rosenblum et al., 2015; Wang et al., 2011). Collectively, these factors provide a more permissive growth environment for NSCs to sprout axons and extend into the host tissue.

5.2.3 Transplantation into multiple SCI models

Another consideration for future experiments includes transplanting self-tracing NSCs into different SCI models. The Tator Lab along with our lab has been credited with establishing the bilateral clip-compression injury model (Rivlin and Tator, 1978). In addition to causing contusion with an adjustable force of clip closure, the model also introduces a 1 min compression to more accurately replicate the clinical mechanism of SCI. We have gone on to characterize the injury model at the cervical (Badner et al., 2016; Satkunendrarajah et al., 2016; Suzuki et al., 2017; Vawda et al., 2019; Wilcox et al., 2014; Zweckberger et al., 2016, 2015), thoracic (Hong et al., 2018; Salewski et al., 2015; Wilcox et al., 2017), and lumbar levels (Moonen et al., 2016) in both rats and mice (Badner et al., 2018). Overall, the model produces clinically relevant functional deficits and reliable pathophysiological mechanisms. Specifically, vascular, immune, and demyelination mechanisms have been best described. This may be a reason why our lab typically sees remyelination effects following NSC transplantation.

Other contusion models also exist, such as the impactor, and produce a similar SCI (Cheriyan et al., 2014). However, complete transection, hemisection or localized transection models are also a popular choice among SCI researchers (Sharif-Alhoseini et al., 2017). While this injury model does not mimic what is seen in clinic, they have been used extensively to investigate axonal regeneration (Dulin et al., 2018; Kadoya et al., 2016; Lu et al., 2017, 2014, 2012, 2003). Transection models have the advantage of selectively lesioning certain sensory or motor pathways, based on the organization of ascending and descending white matter tracts. Our lab may consider transplanting self-tracing NSCs within a dorsal white matter lesion to see the relative contribution of NSCs in DCML and CST regeneration.

5.2.4 Mapping transplant integration in SCI

The purpose of developing self-tracing NSCs is to trace which sensorimotor tracts grafted neurons form neural relays. Stemming from our initial *in vivo* work, using a contusion-compression and/or a transection SCI model, future work should further define the anterograde and retrograde trans-synaptic tracing capabilities of self-tracing NSCs. Tissue clearing and light-sheet microscopy, like what was done by Doerr et al., could be used to view the complete graft in 3D, along with all efferent and afferent connections. Of course, this method would only work with sufficient preservation of GFP and mCherry signalling following tissue clearing procedures.

In addition to localizing traced cells to specific sensory or motor pathways, it would also be interesting to examine the identity of sensory, motor, and interneurons that are forming synaptic connections with the grafts. Dulin et al. have already shown that sensory axons regenerate and localize to graft-derived dorsal horn-like domains (2018). Further excitatory or inhibitory classification of interneurons within these domains can continue to further our understanding of the rewiring occurring within neural circuits after SCI (Maxwell et al., 2007).

5.2.5 Correlation to neurologic recovery

While the bulk of our discussion has focused on the neuroanatomical aspects of neural relay, we should not forget that neural relays are only beneficial if they are able to produce functional recovery. As such, future animal studies should include multiple behavioural assessments to not only capture differences in motor recovery, but also sensory recovery.

In the clinic, SCI patients undergo sensorimotor assessments according to international guidelines (Kirshblum et al., 2011). Clinicians can target specific myotomes and dermatomes corresponding to the patient's level of SCI. Unfortunately, no such standardized procedure has been developed for preclinical research. There exist a multitude of objective tests that have been developed to quantify the progression and recovery of locomotor function in rodent SCI models (Basso, 2004). In order to obtain a comprehensive picture of motor recovery, several behavioural tests should be used together to complement the strengths and limitations of each other (Fouad et al., 2013; Muir and Webb, 2000).

For a cervical C6/7 SCI model, forelimb function is of interest. Forelimb-specific tasks include grip strength (Onifer et al., 1997) and a staircase reaching task (Montoya et al., 1991). Modified reaching tasks have also been developed to distinguish between proper recovery of grasping functions compared to compensatory scooping behaviours (Fenrich et al., 2016). Finer forelimb recovery can also be assessed by the Irvine, Beattie, and Bresnahan (IBB) scale, which requires the evaluation of how rodents manipulate of differently shaped cereals (Irvine et al., 2014).

The C6/7 spinal segments innervate the extensor carpi radialis longus and brevis (wrist extensors), and the triceps (elbow extensors) (Ditunno et al., 1994). Immediately caudal to this, the C8 segment innervates the flexor digitorum profundus (finger flexors) and may also be affected by the injury. These forelimb muscles are responsible for paw placement at the end of a

swing phase and serve to support body weight during stance (Redondo-Castro et al., 2013; Scholle et al., 2001). Thus, some measure of gait is necessary as well. The ladder test has been used to determine skilled walking and coordination of fore- and hindlimbs (Metz and Whishaw, 2009). The CatWalk system can also be used to gather multiple measures of gait, including paw print area, swing speed, swing length, and regularity index (Hamers et al., 2006). However, animals must have sufficient weight-support, determined using the Basso, Beattie, and Bresnahan scale, prior to gait analysis (Barros Filho and Molina, 2008; Basso et al., 1995).

In terms of sensory functional tests, animals are unable to express whether they feel the presence or absence of sensation unless pertaining to pain. In SCI, allodynia can be assessed by performing tail-flick tests or von Frey anaethesiometry (Detloff et al., 2012; Deuis et al., 2017). Allodynia, or decreased pain tolerance, would result in shorter latency times and earlier paw withdrawal.

While sensorimotor recovery is undoubtedly essential in SCI studies, autonomic functions often not reported. Our lab has previously examined respiratory function following SCI and the local neural circuitry within the cervical spinal cord (Satkunendrarajah et al., 2016). Lane et al., have also agreed that respiratory neuroplasticity is also worth considering (Lane et al., 2009). Similarly, other major clinical concerns such as cardiovascular complication bladder dysfunction have not been thoroughly addressed.

5.2.6 Transplantation into other neurologic conditions

Stepping away from SCI, NSCs also have therapeutic potential in other central nervous system conditions, including traumatic brain injury (TBI) and neurodegenerative diseases. TBI pathophysiology shares many similarities to SCI, including the concept of secondary injury mechanisms (Frati et al., 2017). Contusions typically do not cause massive cell death immediately, but widespread neuron and oligodendrocyte loss can be seen over time. Replacement and regeneration of lost cells can still be achieved by NSCs, but the greater complexity in cortical circuits becomes much more important. Not only can sensorimotor function be disturbed, but cognitive deficits may also arise. Thus, self-tracing NSCs may help slowly elucidate the many neuroplastic tracts that are necessary for the recovery of specific functional modalities.

Neuron loss can also be seen in neurodegenerative disorders. Dopaminergic neurons of the substantia nigra are uniquely lost in Parkinson's disease. NSCs have the potential to restore the nigrostriatal pathway and trans-synaptic tracing can be used to confirm appropriate connections have been made. While patients with Alzheimer's disease show global decreases in brain volume, many cases begin with degeneration of the hippocampus. Self-tracing NSCs may be used to investigate whether well-defined hippocampal pathways have been restored. In both diseases, the affected neural circuits are localized which would help with easier tracer detection. However, this may be an overly ambitious proposal given that hypothesized prion mechanisms have deterred many researchers from using NSCs to treat these disorders (Duyckaerts et al., 2019).

Rather than viewing NSCs from a solely therapeutic angle, a developmental neuroscience perspective can be taken to broaden the scope of the use of self-tracing NSCs. Seeding small populations of self-tracing NSCs in neurodevelopmental models can uncover novel circuits and pathways.

5.3 Concluding remarks

SCI research has evolved dramatically in its understanding of secondary injury mechanisms. For a relatively small clinical population, the number of therapies that have reached the level of clinical trials is impressive. Cell-based treatments, such as NSC transplantation, have dominated the field and many researchers have continued to take small steps forward in the search for the best combinatorial treatment to improve functional recovery. However, we believe that in order to make larger advances, we need a better fundamental understanding of both injury mechanisms and treatment mechanisms. It is our hope that our self-tracing NSCs contribute towards this goal in order to evaluate neural relays and relate them to functional outcome measures.

References

Agrawal, S.K., Fehlings, M.G., 1996. Mechanisms of secondary injury to spinal cord axons in vitro: role of Na+, Na (+)-K (+)-ATPase, the Na (+)-H+ exchanger, and the Na (+)-Ca2+ exchanger. J. Neurosci. 16, 545–552.

Agrawal, S.K., Theriault, E., Fehlings, M.G., 1998. Role of Group I Metabotropic Glutamate Receptors in Traumatic Spinal Cord White Matter Injury. J. Neurotrauma 15, 929–941. https://doi.org/10.1089/neu.1998.15.929

Ahmed, Z., Dent, R.G., Suggate, E.L., Barrett, L.B., Seabright, R.J., Berry, M., Logan, A., 2005. Disinhibition of neurotrophin-induced dorsal root ganglion cell neurite outgrowth on CNS myelin by siRNA-mediated knockdown of NgR, p75NTR and Rho-A. Mol. Cell. Neurosci. 28, 509–523. https://doi.org/10.1016/j.mcn.2004.11.002

Ahuja, C.S., Wilson, J.R., Nori, S., Kotter, M.R.N., Druschel, C., Curt, A., Fehlings, M.G., 2017. Traumatic spinal cord injury. Nat. Rev. Dis. Primer 3, 17018. https://doi.org/10.1038/nrdp.2017.18

Alkabie, S., Boileau, A.J., 2016. The Role of Therapeutic Hypothermia After Traumatic Spinal Cord Injury—A Systematic Review. World Neurosurg. 86, 432–449. https://doi.org/10.1016/j.wneu.2015.09.079

Alluin, O., Delivet-Mongrain, H., Gauthier, M.-K., Fehlings, M.G., Rossignol, S., Karimi-Abdolrezaee, S., 2014. Examination of the Combined Effects of Chondroitinase ABC, Growth Factors and Locomotor Training following Compressive Spinal Cord Injury on Neuroanatomical Plasticity and Kinematics. PLoS ONE 9, e111072. https://doi.org/10.1371/journal.pone.0111072

Almad, A., Sahinkaya, F.R., McTigue, D.M., 2011. Oligodendrocyte Fate after Spinal Cord Injury. Neurotherapeutics 8, 262–273. https://doi.org/10.1007/s13311-011-0033-5

Alsanie, W.F., Niclis, J.C., Petratos, S., 2013. Human Embryonic Stem Cell-Derived Oligodendrocytes: Protocols and Perspectives. Stem Cells Dev. 22, 2459–2476. https://doi.org/10.1089/scd.2012.0520

Amemori, T., Ruzicka, J., Romanyuk, N., Jhanwar-Uniyal, M., Sykova, E., Jendelova, P., 2015. Comparison of intraspinal and intrathecal implantation of induced pluripotent stem cell-derived neural precursors for the treatment of spinal cord injury in rats. Stem Cell Res. Ther. 6. https://doi.org/10.1186/s13287-015-0255-2

Anderson, A.J., Cummings, B.J., 2016. Achieving Informed Consent for Cellular Therapies: A Preclinical Translational Research Perspective on Regulations versus a Dose of Reality. J. Law. Med. Ethics 44, 394–401. https://doi.org/10.1177/1073110516667937

Anderson, A.J., Piltti, K.M., Hooshmand, M.J., Nishi, R.A., Cummings, B.J., 2017. Preclinical Efficacy Failure of Human CNS-Derived Stem Cells for Use in the Pathway Study of Cervical Spinal Cord Injury. Stem Cell Rep. 8, 249–263. https://doi.org/10.1016/j.stemcr.2016.12.018

Anderson, K.D., Guest, J.D., Dietrich, W.D., Bartlett Bunge, M., Curiel, R., Dididze, M., Green, B.A., Khan, A., Pearse, D.D., Saraf-Lavi, E., Widerström-Noga, E., Wood, P., Levi, A.D., 2017. Safety of Autologous Human Schwann Cell Transplantation in Subacute Thoracic Spinal Cord Injury. J. Neurotrauma 34, 2950–2963. https://doi.org/10.1089/neu.2016.4895

Anderson, M.A., Burda, J.E., Ren, Y., Ao, Y., O'Shea, T.M., Kawaguchi, R., Coppola, G., Khakh, B.S., Deming, T.J., Sofroniew, M.V., 2016. Astrocyte scar formation aids central nervous system axon regeneration. Nature 532, 195–200. https://doi.org/10.1038/nature17623

Angeli, C.A., Edgerton, V.R., Gerasimenko, Y.P., Harkema, S.J., 2014. Altering spinal cord excitability enables voluntary movements after chronic complete paralysis in humans. Brain 137, 1394–1409. https://doi.org/10.1093/brain/awu038

Arriaga, G., Macopson, J.J., Jarvis, E.D., 2015. Transsynaptic Tracing from Peripheral Targets with Pseudorabies Virus Followed by Cholera Toxin and Biotinylated Dextran Amines Double Labeling. J. Vis. Exp. https://doi.org/10.3791/50672

Arsenijevic, Y., Weiss, S., Schneider, B., Aebischer, P., 2001. Insulin-Like Growth Factor-I Is Necessary for Neural Stem Cell Proliferation and Demonstrates Distinct Actions of Epidermal Growth Factor and Fibroblast Growth Factor-2. J. Neurosci. 21, 7194–7202. https://doi.org/10.1523/JNEUROSCI.21-18-07194.2001

Aschoff, A., Schönitzer, K., 1982. Intra-Axonal Transport of Horseradish Peroxidase (HRP) and Its Use in Neuroanatomy, in: Weiss, D.G., Gorio, A. (Eds.), Axoplasmic Transport in Physiology and Pathology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 167–176. https://doi.org/10.1007/978-3-642-85714-0_23

Assinck, P., Duncan, G.J., Hilton, B.J., Plemel, J.R., Tetzlaff, W., 2017a. Cell transplantation therapy for spinal cord injury. Nat. Neurosci. 20, 637–647. https://doi.org/10.1038/nn.4541

Assinck, P., Duncan, G.J., Plemel, J.R., Lee, M.J., Stratton, J.A., Manesh, S.B., Liu, J., Ramer, L.M., Kang, S.H., Bergles, D.E., Biernaskie, J., Tetzlaff, W., 2017b. Myelinogenic Plasticity of Oligodendrocyte Precursor Cells following Spinal Cord Contusion Injury. J. Neurosci. 37, 8635–8654. https://doi.org/10.1523/JNEUROSCI.2409-16.2017

Badner, A., Hacker, J., Hong, J., Mikhail, M., Vawda, R., Fehlings, M.G., 2018. Splenic involvement in umbilical cord matrix-derived mesenchymal stromal cell-mediated effects following traumatic spinal cord injury. J. Neuroinflammation 15. https://doi.org/10.1186/s12974-018-1243-0

Badner, A., Vawda, R., Laliberte, A., Hong, J., Mikhail, M., Jose, A., Dragas, R., Fehlings, M., 2016. Early Intravenous Delivery of Human Brain Stromal Cells Modulates Systemic Inflammation and Leads to Vasoprotection in Traumatic Spinal Cord Injury: Intravenous Human Brain Stromal Cells in SCI. STEM CELLS Transl. Med. 5, 991–1003. https://doi.org/10.5966/sctm.2015-0295

Banfield, B.W., Kaufman, J.D., Randall, J.A., Pickard, G.E., 2003. Development of Pseudorabies Virus Strains Expressing Red Fluorescent Proteins: New Tools for Multisynaptic Labeling Applications. J. Virol. 77, 10106–10112. https://doi.org/10.1128/JVI.77.18.10106-10112.2003

Barakat, D.J., Gaglani, S.M., Neravetla, S.R., Sanchez, A.R., Andrade, C.M., Pressman, Y., Puzis, R., Garg, M.S., Bunge, M.B., Pearse, D.D., 2005. Survival, Integration, and Axon Growth Support of Glia Transplanted into the Chronically Contused Spinal Cord. Cell Transplant. 14, 225–240. https://doi.org/10.3727/000000005783983106

Barker, R.A., Carpenter, M.K., Forbes, S., Goldman, S.A., Jamieson, C., Murry, C.E., Takahashi, J., Weir, G., 2018. The Challenges of First-in-Human Stem Cell Clinical Trials: What Does This Mean for Ethics and Institutional Review Boards? Stem Cell Rep. 10, 1429–1431. https://doi.org/10.1016/j.stemcr.2018.04.010

Barnett, S.C., Riddell, J.S., 2004. Olfactory ensheathing cells (OECs) and the treatment of CNS injury: advantages and possible caveats. J. Anat. 204, 57–67. https://doi.org/10.1111/j.1469-7580.2004.00257.x

Barthélemy, D., Willerslev-Olsen, M., Lundell, H., Biering-Sørensen, F., Nielsen, J.B., 2015. Assessment of transmission in specific descending pathways in relation to gait and balance following spinal cord injury, in: Progress in Brain Research. Elsevier, pp. 79–101. https://doi.org/10.1016/bs.pbr.2014.12.012

Barros Filho, T.E.P. de, Molina, A.E.I.S., 2008. Analysis of the sensitivity and reproducibility of the Basso, Beattie, Bresnahan (BBB) scale in wistar rats. Clinics 63, 103–108. https://doi.org/10.1590/S1807-59322008000100018

Basso, D.M., 2004. Behavioral Testing After Spinal Cord Injury: Congruities, Complexities, and Controversies. J. Neurotrauma 21, 395–404. https://doi.org/10.1089/089771504323004548

Basso, D.M., Beattie, M.S., Bresnahan, J.C., 1995. A Sensitive and Reliable Locomotor Rating Scale for Open Field Testing in Rats. J. Neurotrauma 12, 1–21. https://doi.org/10.1089/neu.1995.12.1

Bastidas, J., Athauda, G., De La Cruz, G., Chan, W.-M., Golshani, R., Berrocal, Y., Henao, M., Lalwani, A., Mannoji, C., Assi, M., Otero, P.A., Khan, A., Marcillo, A.E., Norenberg, M., Levi, A.D., Wood, P.M., Guest, J.D., Dietrich, W.D., Bartlett Bunge, M., Pearse, D.D., 2017. Human Schwann cells exhibit long-term cell survival, are not tumorigenic and promote repair when transplanted into the contused spinal cord. Glia 65, 1278–1301. https://doi.org/10.1002/glia.23161

Behrman, A.L., Ardolino, E.M., Harkema, S.J., 2017. Activity-based therapy: from basic science to clinical application for recovery after spinal cord injury. J. Neurol. Phys. Ther. JNPT 41, S39.

Beier, K., Cepko, C., 2012. Viral Tracing of Genetically Defined Neural Circuitry. J. Vis. Exp. https://doi.org/10.3791/4253

Bekkers, J.M., 2011. Pyramidal neurons. Curr. Biol. 21, R975. https://doi.org/10.1016/j.cub.2011.10.037

Bernal, A., Arranz, L., 2018. Nestin-expressing progenitor cells: function, identity and therapeutic implications. Cell. Mol. Life Sci. 75, 2177–2195. https://doi.org/10.1007/s00018-018-2794-z

Beyer, F., Küry, P., 2015. Novel approaches for the development of peripheral nerve regenerative therapies. Neural Regen. Res. 10, 1743. https://doi.org/10.4103/1673-5374.170298

Bilsland, L.G., Schiavo, G., 2009. Axonal Transport Tracers, in: Encyclopedia of Neuroscience. Elsevier, pp. 1209–1216. https://doi.org/10.1016/B978-008045046-9.00715-4

Bixby, S., Kruger, G.M., Mosher, J.T., Joseph, N.M., Morrison, S.J., 2002. Cell-Intrinsic Differences between Stem Cells from Different Regions of the Peripheral Nervous System Regulate the Generation of Neural Diversity. Neuron 35, 643–656. https://doi.org/10.1016/S0896-6273(02)00825-5

Bizzini, B., Stoeckel, K., Schwab, M., 1977. AN ANTIGENIC POLYPEPTIDE FRAGMENT ISOLATED FROM TETANUS TOXIN: CHEMICAL CHARACTERIZATION, BINDING TO GANGLIOSIDES AND RETROGRADE AXONAL TRANSPORT IN VARIOUS NEURON SYSTEMS. J. Neurochem. 28, 529–542. https://doi.org/10.1111/j.1471-4159.1977.tb10423.x

Bonizzato, M., Pidpruzhnykova, G., DiGiovanna, J., Shkorbatova, P., Pavlova, N., Micera, S., Courtine, G., 2018. Brain-controlled modulation of spinal circuits improves recovery from spinal cord injury. Nat. Commun. 9. https://doi.org/10.1038/s41467-018-05282-6

Bonner, J.F., Steward, O., 2015. Repair of spinal cord injury with neuronal relays: From fetal grafts to neural stem cells. Brain Res. 1619, 115–123. https://doi.org/10.1016/j.brainres.2015.01.006

Bracken, M.B., Shepard, M.J., Collins, W.F., Holford, T.R., Baskin, D.S., Eisenberg, H.M., Flamm, E., Leo-Summers, L., Maroon, J., Marshall, L.F., Perot Jr., P.I., Piepmeier, J., Sonntag, V.K.H., Wagner, F.C., Wilberger, J.L., Winn, H.R., Young, W., 1992. Methylprednisolone or naloxone treatment after acute spinal cord injury: I-year follow-up data. J. Neurosurg. 76, 23–31.

Bracken, M.B., Shepard, M.J., Collins, W.F., Holford, T.R., Young, W., Baskin, D.S., Eisenberg, H.M., Flamm, E., Leo-Summers, L., Maroon, J., Marshall, L.F., Perot Jr., P.I., Piepmeier, J., Sonntag, V.K.H., Wagner, F.C., Wilberger, J.E., Winn, H.R., 1990. bracken1990.pdf. N. Engl. J. Med. 322, 1405–1411.

Bracken, M.B., Shepard, M.J., Holford, T.R., Leo-Summers, L., Aldrich, E.F., Fazl, M., Fehlings, M., Herr, D.L., Hitchon, P.W., Marshall, L.F., 1997. Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury: results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. Jama 277, 1597–1604.

Braughler, J.M., Hall, E.D., Means, E.D., Waters, T.R., Anderson, D.K., 1987. Evaluation of an intensive methylprednisolone sodium succinate dosing regimen in experimental spinal cord injury. J. Neurosurg. 67, 102–105.

Bregman, B.S., Kunkel-Bagden, E., Schnell, L., Dai, H.N., Gao, D., Schwab, M.E., 1995. Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378, 498–501. https://doi.org/10.1038/378498a0

Brockes, J.P., Fields, K.L., Raff, M.C., 1979. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. Brain Res. 165, 105–118. https://doi.org/10.1016/0006-8993(79)90048-9

Brook, G.A., Plate, D., Franzen, R., Martin, D., Moonen, G., Schoenen, J., Schmitt, A.B., Noth, J., Nacimiento, W., 1998. Spontaneous longitudinally orientated axonal regeneration is associated with the Schwann cell framework within the lesion site following spinal cord compression injury of the rat. J. Neurosci. Res. 53, 51–65. https://doi.org/10.1002/(SICI)1097-4547(19980701)53:1<51::AID-JNR6>3.0.CO;2-I

Buehner, J.J., Forrest, G.F., Schmidt-Read, M., White, S., Tansey, K., Basso, D.M., 2012. Relationship Between ASIA Examination and Functional Outcomes in the NeuroRecovery Network Locomotor Training Program. Arch. Phys. Med. Rehabil. 93, 1530–1540. https://doi.org/10.1016/j.apmr.2012.02.035

Bunge, M.B., Wood, P.M., 2012. Realizing the maximum potential of Schwann cells to promote recovery from spinal cord injury, in: Handbook of Clinical Neurology. Elsevier, pp. 523–540. https://doi.org/10.1016/B978-0-444-52137-8.00032-2

Burney, R.E., Maio, R.F., Maynard, F., Karunas, R., 1993. Incidence, characteristics, and outcome of spinal cord injury at trauma centers in North America. Arch. Surg. 128, 596–599.

Butenschon, J., Zimmermann, T., Schmarowski, N., Nitsch, R., Fackelmeier, B., Friedemann, K., Radyushkin, K., Baumgart, J., Lutz, B., Leschik, J., 2016. PSA-NCAM positive neural progenitors stably expressing BDNF promote functional recovery in a mouse model of spinal cord injury. Stem Cell Res Ther 7, 11. https://doi.org/10.1186/s13287-015-0268-x

Cabot, J.B., Mennone, A., Bogan, N., Carroll, J., Evincer, C., Erichsen, J.T., 1991. Retrograde, trans-synaptic and transneuronal transport of fragment C of tetanus toxin by sympathetic preganglionic neurons. Neuroscience 40, 805–823.

Callaway, E.M., 2008. Transneuronal circuit tracing with neurotropic viruses. Curr. Opin. Neurobiol. 18, 617–623. https://doi.org/10.1016/j.conb.2009.03.007

Calvo, A.C., Oliván, S., Manzano, R., Zaragoza, P., Aguilera, J., Osta, R., 2012. Fragment C of Tetanus Toxin: New Insights into Its Neuronal Signaling Pathway. Int. J. Mol. Sci. 13, 6883–6901. https://doi.org/10.3390/ijms13066883

Capogrosso, M., Milekovic, T., Borton, D., Wagner, F., Moraud, E.M., Mignardot, J.-B., Buse, N., Gandar, J., Barraud, Q., Xing, D., Rey, E., Duis, S., Jianzhong, Y., Ko, W.K.D., Li,

Q., Detemple, P., Denison, T., Micera, S., Bezard, E., Bloch, J., Courtine, G., 2016. A brain–spine interface alleviating gait deficits after spinal cord injury in primates. Nature 539, 284–288. https://doi.org/10.1038/nature20118

Casha, S., Rice, T., Stirling, D.P., Silva, C., Gnanapavan, S., Giovannoni, G., Hurlbert, R.J., Yong, V.W., 2018. Cerebrospinal Fluid Biomarkers in Human Spinal Cord Injury from a Phase II Minocycline Trial. J. Neurotrauma 35, 1918–1928. https://doi.org/10.1089/neu.2018.5899

Casha, S., Zygun, D., McGowan, M.D., Bains, I., Yong, V.W., John Hurlbert, R., 2012. Results of a phase II placebo-controlled randomized trial of minocycline in acute spinal cord injury. Brain 135, 1224–1236. https://doi.org/10.1093/brain/aws072

Catapano, L.A., Magavi, S.S., Macklis, J.D., 2008. Neuroanatomical Tracing of Neuronal Projections with Fluoro-Gold, in: Methods in Molecular Biology. pp. 353–359.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., Studer, L., 2009. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. 27, 275–280. https://doi.org/10.1038/nbt.1529

Chen, B.-Y., Wang, X., Wang, Z.-Y., Wang, Y.-Z., Chen, L.-W., Luo, Z.-J., 2012. Brain-derived neurotrophic factor stimulates proliferation and differentiation of neural stem cells, possibly by triggering the Wnt/β-catenin signaling pathway. J. Neurosci. Res. n/a-n/a. https://doi.org/10.1002/jnr.23138

Chen, K., Liu, J., Assinck, P., Bhatnagar, T., Streijger, F., Zhu, Q., Dvorak, M.F., Kwon, B.K., Tetzlaff, W., Oxland, T.R., 2016. Differential Histopathological and Behavioral Outcomes Eight Weeks after Rat Spinal Cord Injury by Contusion, Dislocation, and Distraction Mechanisms. J. Neurotrauma 33, 1667–1684. https://doi.org/10.1089/neu.2015.4218

Chen, M., Dong, Y., Simard, J.M., 2003. Functional Coupling between Sulfonylurea Receptor Type 1 and a Nonselective Cation Channel in Reactive Astrocytes from Adult Rat Brain. J. Neurosci. 23, 8568–8577. https://doi.org/10.1523/JNEUROSCI.23-24-08568.2003

Chen, M., Ona, V.O., Li, M., Ferrante, R.J., Fink, K.B., Zhu, S., Bian, J., Guo, L., Farrell, L.A., Hersch, S.M., Hobbs, W., Vonsattel, J.-P., Cha, J.-H.J., Friedlander, R.M., 2000. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat. Med. 6, 797–801. https://doi.org/10.1038/77528

Chen, M., Simard, J.M., 2001. Cell Swelling and a Nonselective Cation Channel Regulated by Internal Ca $^{2+}$ and ATP in Native Reactive Astrocytes from Adult Rat Brain. J. Neurosci. 21, 6512–6521. https://doi.org/10.1523/JNEUROSCI.21-17-06512.2001

Chen, M.S., Huber, A.B., van der Haar, M.E., Frank, M., Schnell, L., Spillmann, A.A., Christ, F., Schwab, M.E., 2000. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403, 434–439. https://doi.org/10.1038/35000219

Chen, Y., Tang, Y., Vogel, L., DeVivo, M., 2013. Causes of Spinal Cord Injury. Top. Spinal Cord Inj. Rehabil. 19, 1–8. https://doi.org/10.1310/sci1901-1

Cheng, I., Park, D.Y., Mayle, R.E., Githens, M., Smith, R.L., Park, H.Y., Hu, S.S., Alamin, T.F., Wood, K.B., Kharazi, A.I., 2017. Does timing of transplantation of neural stem cells following spinal cord injury affect outcomes in an animal model? J. Spine Surg. 3, 567–571. https://doi.org/10.21037/jss.2017.10.06

Cheriyan, T., Ryan, D.J., Weinreb, J.H., Cheriyan, J., Paul, J.C., Lafage, V., Kirsch, T., Errico, T.J., 2014. Spinal cord injury models: a review. Spinal Cord 52, 588–595. https://doi.org/10.1038/sc.2014.91

Chew, W.L., Tabebordbar, M., Cheng, J.K.W., Mali, P., Wu, E.Y., Ng, A.H.M., Zhu, K., Wagers, A.J., Church, G.M., 2016. A multifunctional AAV–CRISPR–Cas9 and its host response. Nat. Methods 13, 868–874. https://doi.org/10.1038/nmeth.3993

Coen, L., Osta, R., Maury, M., Brûlet, P., 1997. Construction of hybrid proteins that migrate retrogradely and transynaptically into the central nervous system. Proc. Natl. Acad. Sci. 94, 9400–9405.

Coleman, W.P., Benzel, E., Cahill, D.W., Ducker, T., Geisler, F., Green, B., Gropper, M.R., Goffin, J., Madsen III, P.W., Maiman, D.J., 2000. A critical appraisal of the reporting of the National Acute Spinal Cord Injury Studies (II and III) of methylprednisolone in acute spinal cord injury. Clin. Spine Surg. 13, 185–199.

Craveiro, L.M., Weinmann, O., Roschitzki, B., Gonzenbach, R.R., Zörner, B., Montani, L., Yee, B.K., Feldon, J., Willi, R., Schwab, M.E., 2013. Infusion of anti-Nogo-A antibodies in adult rats increases growth and synapse related proteins in the absence of behavioral alterations. Exp. Neurol. 250, 52–68. https://doi.org/10.1016/j.expneurol.2013.09.015

Craven, B.C., Giangregorio, L.M., Alavinia, S.M., Blencowe, L.A., Desai, N., Hitzig, S.L., Masani, K., Popovic, M.R., 2017. Evaluating the efficacy of functional electrical stimulation therapy assisted walking after chronic motor incomplete spinal cord injury: effects on bone biomarkers and bone strength. J. Spinal Cord Med. 40, 748–758. https://doi.org/10.1080/10790268.2017.1368961

Cripps, R.A., Lee, B.B., Wing, P., Weerts, E., Mackay, J., Brown, D., 2011. A global map for traumatic spinal cord injury epidemiology: towards a living data repository for injury prevention. Spinal Cord 49, 493.

Cruz-Almeida, Y., Felix, E.R., Martinez-Arizala, A., Widerström-Noga, E.G., 2012. Decreased Spinothalamic and Dorsal Column Medial Lemniscus-Mediated Function Is Associated with Neuropathic Pain after Spinal Cord Injury. J. Neurotrauma 29, 2706–2715. https://doi.org/10.1089/neu.2012.2343

Cummings, B.J., Uchida, N., Tamaki, S.J., Anderson, A.J., 2006. Human neural stem cell differentiation following transplantation into spinal cord injured mice: association with recovery of locomotor function. Neurol. Res. 28, 474–481. https://doi.org/10.1179/016164106X115116

Cummings, B.J., Uchida, N., Tamaki, S.J., Salazar, D.L., Hooshmand, M., Summers, R., Gage, F.H., Anderson, A.J., 2005. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc. Natl. Acad. Sci. 102, 14069–14074. https://doi.org/10.1073 pnas.0507063102

Curt, A., Casha, S., Fehlings, M.G., Huhn, S., 2014. Phase I/II Clinical Trial of HuCNS-SC Cells in Chronic Thoracic Spinal Cord Injury.

Cusimano, M., Biziato, D., Brambilla, E., Donegà, M., Alfaro-Cervello, C., Snider, S., Salani, G., Pucci, F., Comi, G., Garcia-Verdugo, J.M., 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. https://doi.org/10.1093/brain/awr339

Dai, X., Park, J.J., Du, Y., Kim, H.R., Wang, G., Errami, Y., Chen, S., 2019. One-step generation of modular CAR-T cells with AAV–Cpf1. Nat. Methods 16, 247–254. https://doi.org/10.1038/s41592-019-0329-7

Daley, G.Q., Hyun, I., Apperley, J.F., Barker, R.A., Benvenisty, N., Bredenoord, A.L., Breuer, C.K., Caulfield, T., Cedars, M.I., Frey-Vasconcells, J., Heslop, H.E., Jin, Y., Lee, R.T., McCabe, C., Munsie, M., Murry, C.E., Piantadosi, S., Rao, M., Rooke, H.M., Sipp, D., Studer, L., Sugarman, J., Takahashi, M., Zimmerman, M., Kimmelman, J., 2016. Setting Global Standards for Stem Cell Research and Clinical Translation: The 2016 ISSCR Guidelines. Stem Cell Rep. 6, 787–797. https://doi.org/10.1016/j.stemcr.2016.05.001

Damak, S., Mosinger, B., Margolskee, R.F., 2008. Transsynaptic transport of wheat germ agglutinin expressed in a subset of type II taste cells of transgenic mice. BMC Neurosci. 9, 96. https://doi.org/10.1186/1471-2202-9-96

De Vivo, M.J., Kartus, P.L., Stover, S.L., Rutt, R.D., Fine, P.R., 1989. Cause of Death for Patients with Spinal Cord Injuries. Arch Intern Med 149, 1761–1766.

Dedeepiya, V.D., William, J.B., Parthiban, J.K., Chidambaram, R., Balamurugan, M., Kuroda, S., Iwasaki, M., Preethy, S., Abraham, S.J., 2014. The known-unknowns in spinal cord injury, with emphasis on cell-based therapies – a review with suggestive arenas for research. Expert Opin. Biol. Ther. 14, 617–634. https://doi.org/10.1517/14712598.2014.889676

Deinhardt, K., Berninghausen, O., Willison, H.J., Hopkins, C.R., Schiavo, G., 2006. Tetanus toxin is internalized by a sequential clathrin-dependent mechanism initiated within lipid microdomains and independent of epsin1. J. Cell Biol. 174, 459–471. https://doi.org/10.1083/jcb.200508170

Detloff, M.R., Fisher, L.C., Deibert, R.J., Basso, D.M., 2012. Acute and Chronic Tactile Sensory Testing after Spinal Cord Injury in Rats. J. Vis. Exp. https://doi.org/10.3791/3247

Deuis, J.R., Dvorakova, L.S., Vetter, I., 2017. Methods Used to Evaluate Pain Behaviors in Rodents. Front. Mol. Neurosci. 10. https://doi.org/10.3389/fnmol.2017.00284

Dietrich, W.D., 2009. Therapeutic hypothermia for spinal cord injury: Crit. Care Med. 37, S238–S242. https://doi.org/10.1097/CCM.0b013e3181aa5d85

Dietrich, W.D., Levi, A.D., Wang, M., Green, B.A., 2011. Hypothermic Treatment for Acute Spinal Cord Injury. Neurotherapeutics 8, 229–239. https://doi.org/10.1007/s13311-011-0035-3

Ditunno, J.F., Young, W., Donovan, W.H., Creasey, G., 1994. The international standards booklet for neurological and functional classification of spinal cord injury. Spinal Cord 32, 70.

Dobkin, B., Apple, D., Barbeau, H., Basso, M., Behrman, A., Deforge, D., Ditunno, J., Dudley, G., Elashoff, R., Fugate, L., Harkema, S., Saulino, M., Scott, M., the Spinal Cord Injury Locomotor Trial (SCILT) Group, 2006. Weight-supported treadmill vs over-ground training for walking after acute incomplete SCI. Neurology 66, 484–493. https://doi.org/10.1212/01.wnl.0000202600.72018.39

Doerr, J., Schwarz, M.K., Wiedermann, D., Leinhaas, A., Jakobs, A., Schloen, F., Schwarz, I., Diedenhofen, M., Braun, N.C., Koch, P., Peterson, D.A., Kubitscheck, U., Hoehn, M., Brüstle, O., 2017. Whole-brain 3D mapping of human neural transplant innervation. Nat. Commun. 8, 14162. https://doi.org/10.1038/ncomms14162

Doetsch, F., Caillé, I., Lim, D.A., García-Verdugo, J.M., Alvarez-Buylla, A., 1999. Subventricular Zone Astrocytes Are Neural Stem Cells in the Adult Mammalian Brain. Cell 97, 703–716. https://doi.org/10.1016/S0092-8674(00)80783-7

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Krause, D.S., Deans, R.J., Keating, A., Prockop, D.J., Horwitz, E.M., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–317. https://doi.org/10.1080/14653240600855905

Doucette, J.R., Kiernan, J.A., Flumerfelt, B.A., 1983. The re-innervation of olfactory glomeruli following transection of primary olfactory axons in the central or peripheral nervous system. J. Anat. 1–19.

Dulin, J.N., Adler, A.F., Kumamaru, H., Poplawski, G.H.D., Lee-Kubli, C., Strobl, H., Gibbs, D., Kadoya, K., Fawcett, J.W., Lu, P., Tuszynski, M.H., 2018. Injured adult motor and sensory axons regenerate into appropriate organotypic domains of neural progenitor grafts. Nat. Commun. 9. https://doi.org/10.1038/s41467-017-02613-x

Duncan, G.J., Manesh, S.B., Hilton, B.J., Assinck, P., Liu, J., Moulson, A., Plemel, J.R., Tetzlaff, W., 2018. Locomotor recovery following contusive spinal cord injury does not require oligodendrocyte remyelination. Nat. Commun. 9. https://doi.org/10.1038/s41467-018-05473-1

Duyckaerts, C., Clavaguera, F., Potier, M.-C., 2019. The prion-like propagation hypothesis in Alzheimer's and Parkinson's disease: Curr. Opin. Neurol. 32, 266–271. https://doi.org/10.1097/WCO.0000000000000672

Eftekharpour, E., Karimi-Abdolrezaee, S., Wang, J., El Beheiry, H., Morshead, C., Fehlings, M.G., 2007. Myelination of Congenitally Dysmyelinated Spinal Cord Axons by Adult Neural Precursor Cells Results in Formation of Nodes of Ranvier and Improved Axonal Conduction. J. Neurosci. 27, 3416–3428. https://doi.org/10.1523/JNEUROSCI.0273-07.2007

Elliott, G., O'Hare, P., 1999. Live-Cell Analysis of a Green Fluorescent Protein-Tagged Herpes Simplex Virus Infection. J VIROL 73, 10.

Enquist, L.W., 2002. Exploiting Circuit-Specific Spread of Pseudorabies Virus in the Central Nervous System: Insights to Pathogenesis and Circuit Tracers. J. Infect. Dis. 186, S209–S214. https://doi.org/10.1086/344278

Evaniew, N., Belley-Côté, E.P., Fallah, N., Noonan, V.K., Rivers, C.S., Dvorak, M.F., 2016. Methylprednisolone for the Treatment of Patients with Acute Spinal Cord Injuries: A Systematic Review and Meta-Analysis. J. Neurotrauma 33, 468–481. https://doi.org/10.1089/neu.2015.4192

Fabian, R.H., Coulter, J.D., 1984. Transneuronal Transport of Lectins. Brain Res. 344, 41–48.

Faulkner, J., Keirstead, H.S., 2005. Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury. Transpl. Immunol. 15, 131–142. https://doi.org/10.1016/j.trim.2005.09.007

Fehlings, M.G., Cadotte, D.W., Fehlings, L.N., 2011a. A Series of Systematic Reviews on the Treatment of Acute Spinal Cord Injury: A Foundation for Best Medical Practice. J. Neurotrauma 28, 1329–1333. https://doi.org/10.1089/neu.2011.1955

Fehlings, M.G., Kim, K.D., Aarabi, B., Rizzo, M., Bond, L.M., McKerracher, L., Vaccaro, A.R., Okonkwo, D.O., 2018. Rho Inhibitor VX-210 in Acute Traumatic Subaxial Cervical Spinal Cord Injury: Design of the SPinal Cord Injury Rho INhibition InvestiGation (SPRING) Clinical Trial. J. Neurotrauma 35, 1049–1056. https://doi.org/10.1089/neu.2017.5434

Fehlings, M.G., Nakashima, H., Nagoshi, N., Chow, D.S.L., Grossman, R.G., Kopjar, B., 2016. Rationale, design and critical end points for the Riluzole in Acute Spinal Cord Injury Study (RISCIS): a randomized, double-blinded, placebo-controlled parallel multi-center trial. Spinal Cord 54, 8.

Fehlings, M.G., Tator, C.H., 1995. The relationships among the severity of spinal cord injury, residual neurological function, axon counts, and counts of retrogradely labeled neurons after experimental spinal cord injury. Exp. Neurol. 132, 220–228.

Fehlings, M.G., Tetreault, L.A., Wilson, J.R., Aarabi, B., Anderson, P., Arnold, P.M., Brodke, D.S., Burns, A.S., Chiba, K., Dettori, J.R., Furlan, J.C., Hawryluk, G., Holly, L.T., Howley, S., Jeji, T., Kalsi-Ryan, S., Kotter, M., Kurpad, S., Marino, R.J., Martin, A.R., Massicotte, E., Merli, G., Middleton, J.W., Nakashima, H., Nagoshi, N., Palmieri, K., Singh, A., Skelly, A.C., Tsai, E.C., Vaccaro, A., Yee, A., Harrop, J.S., 2017a. A Clinical Practice Guideline for the Management of Patients With Acute Spinal Cord Injury and

Central Cord Syndrome: Recommendations on the Timing (≤24 Hours Versus >24 Hours) of Decompressive Surgery. Glob. Spine J. 7, 195S-202S. https://doi.org/10.1177/2192568217706367

Fehlings, M.G., Theodore, N., Harrop, J., Maurais, G., Kuntz, C., Shaffrey, C.I., Kwon, B.K., Chapman, J., Yee, A., Tighe, A., McKerracher, L., 2011b. A Phase I/IIa Clinical Trial of a Recombinant Rho Protein Antagonist in Acute Spinal Cord Injury. J. Neurotrauma 28, 787–796. https://doi.org/10.1089/neu.2011.1765

Fehlings, M.G., Vaccaro, A., Wilson, J.R., Singh, A., W. Cadotte, D., Harrop, J.S., Aarabi, B., Shaffrey, C., Dvorak, M., Fisher, C., Arnold, P., Massicotte, E.M., Lewis, S., Rampersaud, R., 2012. Early versus Delayed Decompression for Traumatic Cervical Spinal Cord Injury: Results of the Surgical Timing in Acute Spinal Cord Injury Study (STASCIS). PLoS ONE 7, e32037. https://doi.org/10.1371/journal.pone.0032037

Fehlings, M.G., Wilson, J.R., Harrop, J.S., Kwon, B.K., Tetreault, L.A., Arnold, P.M., Singh, J.M., Hawryluk, G., Dettori, J.R., 2017b. Efficacy and Safety of Methylprednisolone Sodium Succinate in Acute Spinal Cord Injury: A Systematic Review. Glob. Spine J. 7, 116S-137S. https://doi.org/10.1177/2192568217706366

Felts, P.A., Smith, K.J., 1992. Conduction properties of central nerve fibers remyelinated by Schwann cells. Brain Res. 574, 178–192. https://doi.org/10.1016/0006-8993(92)90815-Q

Fenno, L.E., Mattis, J., Ramakrishnan, C., Hyun, M., Lee, S.Y., He, M., Tucciarone, J., Selimbeyoglu, A., Berndt, A., Grosenick, L., Zalocusky, K.A., Bernstein, H., Swanson, H., Perry, C., Diester, I., Boyce, F.M., Bass, C.E., Neve, R., Huang, Z.J., Deisseroth, K., 2014. Targeting cells with single vectors using multiple-feature Boolean logic. Nat. Methods 11, 763–772. https://doi.org/10.1038/nmeth.2996

Fenrich, K.K., May, Z., Torres-Espín, A., Forero, J., Bennett, D.J., Fouad, K., 2016. Single pellet grasping following cervical spinal cord injury in adult rat using an automated full-time training robot. Behav. Brain Res. 299, 59–71. https://doi.org/10.1016/j.bbr.2015.11.020

Feringa, E.R., Lee, G.W., Vahlsing, H.L., 1985. Cell death in Clarke's column after spinal cord transection. J. Neuropathol. Exp. Neurol. 44, 156–164.

Féron, F., Perry, C., McGrath, J.J., Mackay-Sim, A., 1998. New Techniques for Biopsy and Culture of Human Olfactory Epithelial Neurons. Arch. Otolaryngol. Neck Surg. 124, 861. https://doi.org/10.1001/archotol.124.8.861

Festoff, B.W., Ameenuddin, S., Arnold, P.M., Wong, A., Santacruz, K.S., Citron, B.A., 2006. Minocycline neuroprotects, reduces microgliosis, and inhibits caspase protease expression early after spinal cord injury. J. Neurochem. 97, 1314–1326. https://doi.org/10.1111/j.1471-4159.2006.03799.x

Fitch, M.T., Silver, J., 2008. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp. Neurol. 209, 294–301. https://doi.org/10.1016/j.expneurol.2007.05.014

Fouad, K., Hurd, C., Magnuson, D.S.K., 2013. Functional testing in animal models of spinal cord injury: not as straight forward as one would think. Front. Integr. Neurosci. 7. https://doi.org/10.3389/fnint.2013.00085

Fraher, J.P., 1999. The transitional zone and CNS regeneration. J. Anat. 194, 161–182. https://doi.org/10.1046/j.1469-7580.1999.19420161.x

Francis, J.E., Mason, D., Lévy, R., 2017. Evaluation of quantum dot conjugated antibodies for immunofluorescent labelling of cellular targets. Beilstein J. Nanotechnol. 8, 1238–1249. https://doi.org/10.3762/bjnano.8.125

Frati, A., Cerretani, D., Fiaschi, A., Frati, P., Gatto, V., La Russa, R., Pesce, A., Pinchi, E., Santurro, A., Fraschetti, F., Fineschi, V., 2017. Diffuse Axonal Injury and Oxidative Stress: A Comprehensive Review. Int. J. Mol. Sci. 18, 2600. https://doi.org/10.3390/ijms18122600

Freund, P., Schmidlin, E., Wannier, T., Bloch, J., Mir, A., Schwab, M.E., Rouiller, E.M., 2006. Nogo-A–specific antibody treatment enhances sprouting and functional recovery after cervical lesion in adult primates. Nat. Med. 12, 790–792. https://doi.org/10.1038/nm1436

Freund, P., Wannier, T., Schmidlin, E., Bloch, J., Mir, A., Schwab, M.E., Rouiller, E.M., 2007. Anti-Nogo-A antibody treatment enhances sprouting of corticospinal axons rostral to a unilateral cervical spinal cord lesion in adult macaque monkey. J. Comp. Neurol. 502, 644–659. https://doi.org/10.1002/cne.21321

Freund, P., Weiskopf, N., Ashburner, J., Wolf, K., Sutter, R., Altmann, D.R., Friston, K., Thompson, A., Curt, A., 2013. MRI investigation of the sensorimotor cortex and the corticospinal tract after acute spinal cord injury: a prospective longitudinal study. Lancet Neurol. 12, 873–881. https://doi.org/10.1016/S1474-4422(13)70146-7

Gage, F.H., 2000. Mammalian Neural Stem Cells. Science 287, 1433–1438. https://doi.org/10.1126/science.287.5457.1433

Garrido-Mesa, N., Zarzuelo, A., Gálvez, J., 2013. Minocycline: far beyond an antibiotic: Minocycline: far beyond an antibiotic. Br. J. Pharmacol. 169, 337–352. https://doi.org/10.1111/bph.12139

Ge, X., Xi, H., Yang, F., Zhi, X., Fu, Y., Chen, D., Xu, R.-H., Lin, G., Qu, J., Zhao, J., Gu, F., 2016. CRISPR/Cas9-AAV Mediated Knock-in at NRL Locus in Human Embryonic Stem Cells. Mol. Ther. - Nucleic Acids 5, e393. https://doi.org/10.1038/mtna.2016.100

Geffner, L.F., Santacruz, P., Izurieta, M., Flor, L., Maldonado, B., Auad, A.H., Montenegro, X., Gonzalez, R., Silva, F., 2008. Administration of Autologous Bone Marrow Stem Cells into Spinal Cord Injury Patients via Multiple Routes is Safe and Improves their Quality of Life: Comprehensive Case Studies. Cell Transplant. 17, 1277–1293. https://doi.org/10.3727/096368908787648074

Ghobrial, G.M., Anderson, K.D., Dididze, M., Martinez-Barrizonte, J., Sunn, G.H., Gant, K.L., Levi, A.D., 2017. Human Neural Stem Cell Transplantation in Chronic Cervical Spinal

Cord Injury: Functional Outcomes at 12 Months in a Phase II Clinical Trial. Neurosurgery 64, 87–91. https://doi.org/10.1093/neuros/nyx242

Giacci, M., Fitzgerald, M., 2018. Oligodendroglia Are Particularly Vulnerable to Oxidative Damage After Neurotrauma In Vivo. J. Exp. Neurosci. 12, 1–3. https://doi.org/10.1177/1179069518810004

Ginis, K.M., Hicks, A.L., Latimer, A.E., Warburton, D.E.R., Bourne, C., Ditor, D.S., Goodwin, D.L., Hayes, K.C., McCartney, N., McIlraith, A., 2011. The development of evidence-informed physical activity guidelines for adults with spinal cord injury. Spinal Cord 49, 1088.

Gonzenbach, R.R., Zoerner, B., Schnell, L., Weinmann, O., Mir, A.K., Schwab, M.E., 2012. Delayed Anti-Nogo-A Antibody Application after Spinal Cord Injury Shows Progressive Loss of Responsiveness. J. Neurotrauma 29, 567–578. https://doi.org/10.1089/neu.2011.1752

Goshgarian, H.G., Buttry, J.L., 2014. The pattern and extent of retrograde transsynaptic transport of WGA-Alexa 488 in the phrenic motor system is dependent upon the site of application. J. Neurosci. Methods 222, 156–164. https://doi.org/10.1016/j.jneumeth.2013.11.003

Gossen, M., Bujard, H., 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. 89, 5547–5551. https://doi.org/10.1073/pnas.89.12.5547

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., Bujard, H., 1995. Transcriptional activation by tetracyclines in mammalian cells. Science 268, 1766–1769. https://doi.org/10.1126/science.7792603

GrandPré, T., Nakamura, F., Vartanian, T., Strittmatter, S.M., 2000. Identi®cation of the Nogo inhibitor of axon regeneration as a Reticulon protein 403, 6.

Gritti, A., Parati, E., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morassutti, D., Roisen, F., Nickel, D., Vescovi, A., 1996. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J. Neurosci. 16, 1091–1100. https://doi.org/10.1523/JNEUROSCI.16-03-01091.1996

Grossman, R.G., Fehlings, M.G., Frankowski, R.F., Burau, K.D., Chow, D.S.L., Tator, C., Teng, A., Toups, E.G., Harrop, J.S., Aarabi, B., Shaffrey, C.I., Johnson, M.M., Harkema, S.J., Boakye, M., Guest, J.D., Wilson, J.R., 2014. A Prospective, Multicenter, Phase I Matched-Comparison Group Trial of Safety, Pharmacokinetics, and Preliminary Efficacy of Riluzole in Patients with Traumatic Spinal Cord Injury. J. Neurotrauma 31, 239–255. https://doi.org/10.1089/neu.2013.2969

Guenard, V., Gwynn, L., Wood, P., 1994. Astrocytes inhibit Schwann cell proliferation and myelination of dorsal root ganglion neurons in vitro. J. Neurosci. 14, 2980–2992. https://doi.org/10.1523/JNEUROSCI.14-05-02980.1994

Hachem, L.D., Ahuja, C.S., Fehlings, M.G., 2017. Assessment and management of acute spinal cord injury: From point of injury to rehabilitation. J. Spinal Cord Med. 40, 665–675. https://doi.org/10.1080/10790268.2017.1329076

Hadley, M.N., Walters, B.C., Grabb, P.A., Oyesiku, N.M., Przybylski, G.J., Resnick, D.K., Ryken, T.C., Mielke, D.H., 2002. Guidelines for the management of acute cervical spine and spinal cord injuries., in: Clinical Neurosurgery. Congress of Neurological Surgeons, pp. 407–498.

Hall, E.D., Braughler, J.M., 1982. Effects of intravenous methylprednisolone on spinal cord lipid peroxidation and (Na++ K+)-ATPase activity: Dose-response analysis during 1st hour after contusion injury in the cat. J. Neurosurg. 57, 247–253.

Halpern, J.L., Habig, W.H., Neale, E.A., Stibitz, S., 1990. Cloning and expression of functional fragment C of tetanus toxin. Infect. Immun. 58, 1004–1009.

Hamers, F.P.T., Koopmans, G.C., Joosten, E.A.J., 2006. CatWalk-Assisted Gait Analysis in the Assessment of Spinal Cord Injury. J. Neurotrauma 23, 537–548. https://doi.org/10.1089/neu.2006.23.537

Hamilton, L.K., Truong, M.K.V., Bednarczyk, M.R., Aumont, A., Fernandes, K.J.L., 2009. Cellular organization of the central canal ependymal zone, a niche of latent neural stem cells in the adult mammalian spinal cord. Neuroscience 164, 1044–1056. https://doi.org/10.1016/j.neuroscience.2009.09.006

Harvey, L.A., Glinsky, J.V., Bowden, J.L., 2016. The effectiveness of 22 commonly administered physiotherapy interventions for people with spinal cord injury: a systematic review. Spinal Cord 54, 914.

Harvey, P.J., Grochmal, J., Tetzlaff, W., Gordon, T., Bennett, D.J., 2005. An investigation into the potential for activity-dependent regeneration of the rubrospinal tract after spinal cord injury. Eur. J. Neurosci. 22, 3025–3035. https://doi.org/10.1111/j.1460-9568.2005.04514.x

Hawryluk, G., Whetstone, W., Saigal, R., Ferguson, A., Talbott, J., Bresnahan, J., Dhall, S., Pan, J., Beattie, M., Manley, G., 2015. Mean Arterial Blood Pressure Correlates with Neurological Recovery after Human Spinal Cord Injury: Analysis of High Frequency Physiologic Data. J. Neurotrauma 32, 1958–1967. https://doi.org/10.1089/neu.2014.3778

Hawryluk, G.W.J., Mothe, A., Wang, J., Wang, S., Tator, C., Fehlings, M.G., 2012. An In Vivo Characterization of Trophic Factor Production Following Neural Precursor Cell or Bone Marrow Stromal Cell Transplantation for Spinal Cord Injury. Stem Cells Dev. 21, 2222–2238. https://doi.org/10.1089/scd.2011.0596

Herrmann, J.E., Imura, T., Song, B., Qi, J., Ao, Y., Nguyen, T.K., Korsak, R.A., Takeda, K., Akira, S., Sofroniew, M.V., 2008. STAT3 is a Critical Regulator of Astrogliosis and Scar Formation after Spinal Cord Injury. J. Neurosci. 28, 7231–7243. https://doi.org/10.1523/JNEUROSCI.1709-08.2008

Hong, J., Chang, A., Zavvarian, M-M., Wang, J., Liu, Y., Fehlings., M., 2018. Level-specific differences in systemic expression of pro- and anti-inflammatory cytokines and chemokines after spinal cord injury. Int. J. Molec. Sci. 19, 2167

Honmou, O., Felts, P.A., Waxman, S.G., Kocsis, J.D., 1996. Restoration of Normal Conduction Properties in Demyelinated Spinal Cord Axons in the Adult Rat by Transplantation of Exogenous Schwann Cells. J. Neurosci. 16, 3199–3208. https://doi.org/10.1523/JNEUROSCI.16-10-03199.1996

Hoshino, K., Horie, M., Nagy, A., Berényi, A., Benedek, G., Norita, M., 2010. Direct synaptic connections between superior colliculus afferents and thalamo-insular projection neurons in the feline suprageniculate nucleus: A double-labeling study with WGA-HRP and kainic acid. Neurosci. Res. 66, 7–13. https://doi.org/10.1016/j.neures.2009.09.002

Hosseini, S.M., Sani, M., Haider, Kh.H., Dorvash, M., Ziaee, S.M., Karimi, A., Namavar, M.R., 2018. Concomitant use of mesenchymal stem cells and neural stem cells for treatment of spinal cord injury: A combo cell therapy approach. Neurosci. Lett. 668, 138–146. https://doi.org/10.1016/j.neulet.2018.01.008

Hugenholtz, H., 2003. Methylprednisolone for acute spinal cord injury: not a standard of care. CMAJ 168, 1145–1146.

Huh, Y., Oh, M.S., Leblanc, P., Kim, K.-S., 2010. Gene transfer in the nervous system and implications for transsynaptic neuronal tracing. Expert Opin. Biol. Ther. 10, 763–772. https://doi.org/10.1517/14712591003796538

Hwang, D.H., Shin, H.Y., Kwon, M.J., Choi, J.Y., Ryu, B.Y., Kim, B.G., 2014. Survival of neural stem cell grafts in the lesioned spinal cord is enhanced by a combination of treadmill locomotor training via insulin-like growth factor-1 signaling. J Neurosci 34, 12788–800. https://doi.org/10.1523/jneurosci.5359-13.2014

Hwang, I., Hahm, S.-C., Choi, K.-A., Park, S.-H., Jeong, H., Yea, J.-H., Kim, J., Hong, S., 2016. Intrathecal Transplantation of Embryonic Stem Cell-Derived Spinal GABAergic Neural Precursor Cells Attenuates Neuropathic Pain in a Spinal Cord Injury Rat Model. Cell Transplant. 25, 593–607. https://doi.org/10.3727/096368915X689460

Iida, T., Iwanami, A., Sanosaka, T., Kohyama, J., Miyoshi, H., Nagoshi, N., Kashiwagi, R., Toyama, Y., Matsumoto, M., Nakamura, M., Okano, H., 2017. Whole-Genome DNA Methylation Analyses Revealed Epigenetic Instability in Tumorigenic Human iPS Cell-Derived Neural Stem/Progenitor Cells: Epigenetic Instability in hiPSC-NS/PCs. STEM CELLS 35, 1316–1327. https://doi.org/10.1002/stem.2581

Ikegami, T., Nakamura, M., Yamane, J., Katoh, H., Okada, S., Iwanami, A., Watanabe, K., Ishii, K., Kato, F., Fujita, H., Takahashi, T., Okano, H.J., Toyama, Y., Okano, H., 2005. Chondroitinase ABC combined with neural stem/progenitor cell transplantation enhances graft cell migration and outgrowth of growth-associated protein-43-positive fibers after rat spinal cord injury. Eur. J. Neurosci. 22, 3036–3046. https://doi.org/10.1111/j.1460-9568.2005.04492.x

Irvine, K.-A., Ferguson, A.R., Mitchell, K.D., Beattie, S.B., Lin, A., Stuck, E.D., Huie, J.R., Nielson, J.L., Talbott, J.F., Inoue, T., Beattie, M.S., Bresnahan, J.C., 2014. The Irvine, Beatties, and Bresnahan (IBB) Forelimb Recovery Scale: An Assessment of Reliability and Validity. Front. Neurol. 5. https://doi.org/10.3389/fneur.2014.00116

Itaya, S.K., Van Hoesen, G.W., 1982. WGA-HRP as a transneuronal marker in the visual pathways of monkey and rat. Brain Res. 236, 199–204. https://doi.org/10.1016/0006-8993(82)90046-4

Jain, N.B., Ayers, G.D., Peterson, E.N., Harris, M.B., Morse, L., O'Connor, K.C., Garshick, E., 2015. Traumatic Spinal Cord Injury in the United States, 1993-2012. JAMA 313, 2236. https://doi.org/10.1001/jama.2015.6250

Jiao, G., Lou, G., Mo, Y., Pan, Y., Zhang, Z., Guo, R., Li, Z., 2017. A combination of GDNF and hUCMSC transplantation loaded on SF/AGs composite scaffolds for spinal cord injury repair. Mater. Sci. Eng. C 74, 230–237. https://doi.org/10.1016/j.msec.2016.12.017

Johansson, C.B., Momma, S., Clarke, D.L., Risling, M., Lendahl, U., Frisén, J., 1999. Identification of a Neural Stem Cell in the Adult Mammalian Central Nervous System. Cell 96, 25–34. https://doi.org/10.1016/S0092-8674(00)80956-3

Jones, L.A.T., Bryden, A., Wheeler, T.L., Tansey, K.E., Anderson, K.D., Beattie, M.S., Blight, A., Curt, A., Field-Fote, E., Guest, J.D., Hseih, J., Jakeman, L.B., Kalsi-Ryan, S., Krisa, L., Lammertse, D.P., Leiby, B., Marino, R., Schwab, J.M., Scivoletto, G., Tulsky, D.S., Wirth, E., Zariffa, J., Kleitman, N., Mulcahey, M.J., Steeves, J.D., 2018. Considerations and recommendations for selection and utilization of upper extremity clinical outcome assessments in human spinal cord injury trials. Spinal Cord 56, 414–425. https://doi.org/10.1038/s41393-017-0015-5

Kadoya, K., Lu, P., Nguyen, K., Lee-Kubli, C., Kumamaru, H., Yao, L., Knackert, J., Poplawski, G., Dulin, J.N., Strobl, H., Takashima, Y., Biane, J., Conner, J., Zhang, S.-C., Tuszynski, M.H., 2016. Spinal cord reconstitution with homologous neural grafts enables robust corticospinal regeneration. Nat. Med. 22, 479–487. https://doi.org/10.1038/nm.4066

Kanno, H., Pearse, D.D., Ozawa, H., Itoi, E., Bunge, M.B., 2015. Schwann cell transplantation for spinal cord injury repair: its significant therapeutic potential and prospectus. Rev. Neurosci. 26. https://doi.org/10.1515/revneuro-2014-0068

Kapadia, N., Masani, K., Catharine Craven, B., Giangregorio, L.M., Hitzig, S.L., Richards, K., Popovic, M.R., 2014. A randomized trial of functional electrical stimulation for walking in incomplete spinal cord injury: Effects on walking competency. J. Spinal Cord Med. 37, 511–524. https://doi.org/10.1179/2045772314Y.0000000263

Kapadia, N., Zivanovic, V., Popovic, M., 2013. Restoring Voluntary Grasping Function in Individuals with Incomplete Chronic Spinal Cord Injury: Pilot Study. Top. Spinal Cord Inj. Rehabil. 19, 279–287. https://doi.org/10.1310/sci1904-279

Karibe, H., Zarow, G.J., Graham, S.H., Weinstein, P.R., 1994. Mild Intraischemic Hypothermia Reduces Postischemic Hyperperfusion, Delayed Postischemic Hypoperfusion, Blood-

Brain Barrier Disruption, Brain Edema, and Neuronal Damage Volume after Temporary Focal Cerebral Ischemia in Rats. J. Cereb. Blood Flow Metab. 14, 620–627. https://doi.org/10.1038/jcbfm.1994.77

Karimi-Abdolrezaee, S., 2006. Delayed Transplantation of Adult Neural Precursor Cells Promotes Remyelination and Functional Neurological Recovery after Spinal Cord Injury. J. Neurosci. 26, 3377–3389. https://doi.org/10.1523/JNEUROSCI.4184-05.2006

Karimi-Abdolrezaee, S., Schut, D., Wang, J., Fehlings, M.G., 2012. Chondroitinase and Growth Factors Enhance Activation and Oligodendrocyte Differentiation of Endogenous Neural Precursor Cells after Spinal Cord Injury. PLoS ONE 7, e37589. https://doi.org/10.1371/journal.pone.0037589

Kawabata, S., Takano, M., Numasawa-Kuroiwa, Y., Itakura, G., Kobayashi, Y., Nishiyama, Y., Sugai, K., Nishimura, S., Iwai, H., Isoda, M., Shibata, S., Kohyama, J., Iwanami, A., Toyama, Y., Matsumoto, M., Nakamura, M., Okano, H., 2016. Grafted Human iPS Cell-Derived Oligodendrocyte Precursor Cells Contribute to Robust Remyelination of Demyelinated Axons after Spinal Cord Injury. Stem Cell Rep. 6, 1–8. https://doi.org/10.1016/j.stemcr.2015.11.013

Kayalioglu, G., 2009. Projections from the Spinal Cord to the Brain, in: The Spinal Cord. Elsevier, pp. 148–167. https://doi.org/10.1016/B978-0-12-374247-6.50014-6

Keirstead, H.S., Nistor, G.I., Bernal, G., Totoiu, M.O., Cloutier, F., Sharp, K., Oswald, S., 2005. Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cell Transplants Remyelinate and Restore Locomotion after Spinal Cord Injury. J. Neurosci. 25, 4694–4705. https://doi.org/10.1523/JNEUROSCI.0311-05.2005

Kerr, C.L., Letzen, B.S., Hill, C.M., Agrawal, G., Thakor, N.V., Sterneckert, J.L., Gearhart, J.D., All, A.H., 2010. Efficient Differentiation of Human Embryonic Stem Cells into Oligodendrocyte Progenitors for Application in a Rat Contusion Model of Spinal Cord Injury. Int. J. Neurosci. 120, 305–313. https://doi.org/10.3109/00207450903585290

Kim, E.J., Jacobs, M.W., Ito-Cole, T., Callaway, E.M., 2016. Improved Monosynaptic Neural Circuit Tracing Using Engineered Rabies Virus Glycoproteins. Cell Rep. 15, 692–699. https://doi.org/10.1016/j.celrep.2016.03.067

Kim, J.B., Lee, H., Araúzo-Bravo, M.J., Hwang, K., Nam, D., Park, M.R., Zaehres, H., Park, K.I., Lee, S., 2015. Oct4-induced oligodendrocyte progenitor cells enhance functional recovery in spinal cord injury model. EMBO J. 34, 2971–2983. https://doi.org/10.15252/embj.201592652

Kim, K., Doi, A., Wen, B., Ng, K., Zhao, R., Cahan, P., Kim, J., Aryee, M.J., Ji, H., Ehrlich, L.I.R., Yabuuchi, A., Takeuchi, A., Cunniff, K.C., Hongguang, H., Mckinney-Freeman, S., Naveiras, O., Yoon, T.J., Irizarry, R.A., Jung, N., Seita, J., Hanna, J., Murakami, P., Jaenisch, R., Weissleder, R., Orkin, S.H., Weissman, I.L., Feinberg, A.P., Daley, G.Q., 2010. Epigenetic memory in induced pluripotent stem cells. Nature 467, 285–290. https://doi.org/10.1038/nature09342

Kirshblum, S.C., Burns, S.P., Biering-Sorensen, F., Donovan, W., Graves, D.E., Jha, A., Johansen, M., Jones, L., Krassioukov, A., Mulcahey, M.J., Schmidt-Read, M., Waring, W., 2011. International standards for neurological classification of spinal cord injury (Revised 2011). J. Spinal Cord Med. 34, 535–546. https://doi.org/10.1179/204577211X13207446293695

Kissa, K., Mordelet, E., Soudais, C., Kremer, E.J., Demeneix, B.A., Brûlet, P., Coen, L., 2002. In Vivo Neuronal Tracing with GFP-TTC Gene Delivery. Mol. Cell. Neurosci. 20, 627–637. https://doi.org/10.1006/mcne.2002.1141

Kitzman, P.H., 2009. Effectiveness of riluzole in suppressing spasticity in the spinal cord injured rat. Neurosci. Lett. 455, 150–153. https://doi.org/10.1016/j.neulet.2009.03.016

Klein, R., Ruttkowski, B., Knapp, E., Salmons, B., Günzburg, W.H., Hohenadl, C., 2006. WPRE-mediated enhancement of gene expression is promoter and cell line specific. Gene 372, 153–161. https://doi.org/10.1016/j.gene.2005.12.018

Kloos, A., Fisher, L., Detloff, M., Hassenzahl, D., Basso, D., 2005. Stepwise motor and all-or-none sensory recovery is associated with nonlinear sparing after incremental spinal cord injury in rats. Exp. Neurol. 191, 251–265. https://doi.org/10.1016/j.expneurol.2004.09.016

Kobayashi, Y., Okada, Y., Itakura, G., Iwai, H., Nishimura, S., Yasuda, A., Nori, S., Hikishima, K., Konomi, T., Fujiyoshi, K., Tsuji, O., Toyama, Y., Yamanaka, S., Nakamura, M., Okano, H., 2012. Pre-Evaluated Safe Human iPSC-Derived Neural Stem Cells Promote Functional Recovery after Spinal Cord Injury in Common Marmoset without Tumorigenicity. PLoS ONE 7, e52787. https://doi.org/10.1371/journal.pone.0052787

Krause, J.S., Reed, K.S., McArdle, J.J., 2010. A structural analysis of health outcomes after spinal cord injury. J. Spinal Cord Med. 33, 22–32.

Krueger, H., Noonan, V.K., Trenaman, L.M., Joshi, P., Rivers, C.S., 2013. The economic burden of traumatic spinal cord injury in Canada. Chronic Dis. Inj. Can. 33, 113–122.

Kucher, K., Johns, D., Maier, D., Abel, R., Badke, A., Baron, H., Thietje, R., Casha, S., Meindl, R., Gomez-Mancilla, B., Pfister, C., Rupp, R., Weidner, N., Mir, A., Schwab, M.E., Curt, A., 2018. First-in-Man Intrathecal Application of Neurite Growth-Promoting Anti-Nogo-A Antibodies in Acute Spinal Cord Injury. Neurorehabil. Neural Repair 32, 578–589. https://doi.org/10.1177/1545968318776371

Kwon, B.K., Fisher, C.G., Dvorak, M.F., Tetzlaff, W., 2005. Strategies to Promote Neural Repair and Regeneration After Spinal Cord Injury: Spine 30, S3–S13. https://doi.org/10.1097/01.brs.0000175186.17923.87

Kwon, B.K., Liu, J., Oschipok, L., Teh, J., Liu, Z.W., Tetzlaff, W., 2004. Rubrospinal neurons fail to respond to brain-derived neurotrophic factor applied to the spinal cord injury site 2 months after cervical axotomy. Exp. Neurol. 189, 45–57. https://doi.org/10.1016/j.expneurol.2004.05.034

Kwon, B.K., Streijger, F., Hill, C.E., Anderson, A.J., Bacon, M., Beattie, M.S., Blesch, A., Bradbury, E.J., Brown, A., Bresnahan, J.C., Case, C.C., Colburn, R.W., David, S., Fawcett, J.W., Ferguson, A.R., Fischer, I., Floyd, C.L., Gensel, J.C., Houle, J.D., Jakeman, L.B., Jeffery, N.D., Jones, L.A.T., Kleitman, N., Kocsis, J., Lu, P., Magnuson, D.S.K., Marsala, M., Moore, S.W., Mothe, A.J., Oudega, M., Plant, G.W., Rabchevsky, A.S., Schwab, J.M., Silver, J., Steward, O., Xu, X.-M., Guest, J.D., Tetzlaff, W., 2015. Large animal and primate models of spinal cord injury for the testing of novel therapies. Exp. Neurol. 269, 154–168. https://doi.org/10.1016/j.expneurol.2015.04.008

Lai, B.-Q., Qiu, X.-C., Zhang, K., Zhang, R.-Y., Jin, H., Li, G., Shen, H.-Y., Wu, J.-L., Ling, E.-A., Zeng, Y.-S., 2015. Cholera Toxin B Subunit Shows Transneuronal Tracing after Injection in an Injured Sciatic Nerve. PLOS ONE 10, e0144030. https://doi.org/10.1371/journal.pone.0144030

Lakatos, A., Franklin, R.J.M., Barnett, S.C., 2000. Olfactory ensheathing cells and Schwann cells differ in their in vitro interactions with astrocytes. Glia 32, 214–225. https://doi.org/10.1002/1098-1136(200012)32:3<214::AID-GLIA20>3.0.CO;2-7

Lanciego, J.L., Wouterlood, F.G., Erro, E., Giménez-Amaya, J.M., 1998. Multiple axonal tracing: simultaneous detection of three tracers in the same section. Histochem. Cell Biol. 110, 509. https://doi.org/10.1007/s004180050312

Lane, M.A., Lee, K.-Z., Fuller, D.D., Reier, P.J., 2009. Spinal circuitry and respiratory recovery following spinal cord injury. Respir. Physiol. Neurobiol. 169, 123–132. https://doi.org/10.1016/j.resp.2009.08.007

Lane, M.A., Lepore, A.C., Fischer, I., 2017. Improving the therapeutic efficacy of neural progenitor cell transplantation following spinal cord injury. Expert Rev. Neurother. 17, 433–440. https://doi.org/10.1080/14737175.2017.1270206

Lazarov, N.E., 2013. Neuroanatomical Tract-Tracing Using Biotinylated Dextran Amine, in: Zhou, R., Mei, L. (Eds.), Neural Development. Humana Press, Totowa, NJ, pp. 323–334. https://doi.org/10.1007/978-1-62703-444-9_30

Lee, S.M., Yune, T.Y., Kim, S.J., Park, D.W., Lee, Y.K., Kim, Y.C., Oh, Y.J., Markelonis, G.J., Oh, T.H., 2003. Minocycline Reduces Cell Death and Improves Functional Recovery after Traumatic Spinal Cord Injury in the Rat. J. Neurotrauma 20, 1017–1027. https://doi.org/10.1089/089771503770195867

Levi, A.D., Anderson, K.D., Okonkwo, D.O., Park, P., Bryce, T.N., Kurpad, S.N., Aarabi, B., Hsieh, J., Gant, K., 2019. Clinical Outcomes from a Multi-Center Study of Human Neural Stem Cell Transplantation in Chronic Cervical Spinal Cord Injury. J. Neurotrauma 36, 891–902. https://doi.org/10.1089/neu.2018.5843

Levi, A.D., Okonkwo, D.O., Park, P., Jenkins, A.L., Kurpad, S.N., Parr, A.M., Ganju, A., Aarabi, B., Kim, D., Casha, S., Fehlings, M.G., Harrop, J.S., Anderson, K.D., Gage, A., Hsieh, J., Huhn, S., Curt, A., Guzman, R., 2017. Emerging Safety of Intramedullary Transplantation of Human Neural Stem Cells in Chronic Cervical and Thoracic Spinal Cord Injury. Neurosurgery 82, 562–575. https://doi.org/10.1093/neuros/nyx250

Levi, L., Wolf, A., Belzberg, H., 1993. Hemodynamic parameters in patients with acute cervical cord trauma: description, intervention, and prediction of outcome. Neurosurgery 33, 1007–1017.

Levine, J.M., Reynolds, R., Fawcett, J.W., 2001. The oligodendrocyte precursor cell in health and disease. Trends Neurosci. 24, 39–47. https://doi.org/10.1016/S0166-2236(00)01691-X

Levy, S.L., White, J.J., Sillitoe, R.V., 2015. Wheat Germ Agglutinin (WGA) Tracing: A Classic Approach for Unraveling Neural Circuitry, in: Arenkiel, B.R. (Ed.), Neural Tracing Methods. Springer New York, New York, NY, pp. 51–66. https://doi.org/10.1007/978-1-4939-1963-5_2

Li, W., Cai, W.Q., Li, C.R., 2006. Repair of spinal cord injury by neural stem cells modified with BDNF gene in rats. Neurosci Bull 22, 34–40.

Li, X., Burnight, E.R., Cooney, A.L., Malani, N., Brady, T., Sander, J.D., Staber, J., Wheelan, S.J., Joung, J.K., McCray, P.B., Bushman, F.D., Sinn, P.L., Craig, N.L., 2013. piggyBac transposase tools for genome engineering. Proc. Natl. Acad. Sci. 110, E2279–E2287. https://doi.org/10.1073/pnas.1305987110

Li, Z., Guo, G.-H., Wang, G.-S., Guan, C.-X., Yue, L., 2014. Influence of neural stem cell transplantation on angiogenesis in rats with spinal cord injury. Genet. Mol. Res. 13, 6083–6092. https://doi.org/10.4238/2014.August.7.23

Libbrecht, S., Van den Haute, C., Malinouskaya, L., Gijsbers, R., Baekelandt, V., 2017. Evaluation of WGA–Cre-dependent topological transgene expression in the rodent brain. Brain Struct. Funct. 222, 717–733. https://doi.org/10.1007/s00429-016-1241-x

Liebscher, T., Schnell, L., Schnell, D., Scholl, J., Schneider, R., Gullo, M., Fouad, K., Mir, A., Rausch, M., Kindler, D., Hamers, F.P.T., Schwab, M.E., 2005. Nogo-A antibody improves regeneration and locomotion of spinal cord-injured rats. Ann. Neurol. 58, 706–719. https://doi.org/10.1002/ana.20627

Liu, S., Sandner, B., Schackel, T., Nicholson, L., Chtarto, A., Tenenbaum, L., Puttagunta, R., Müller, R., Weidner, N., Blesch, A., 2017. Regulated viral BDNF delivery in combination with Schwann cells promotes axonal regeneration through capillary alginate hydrogels after spinal cord injury. Acta Biomater. 60, 167–180. https://doi.org/10.1016/j.actbio.2017.07.024

Liu, S.-L., Zhang, Z.-L., Sun, E.-Z., Peng, J., Xie, M., Tian, Z.-Q., Lin, Y., Pang, D.-W., 2011. Visualizing the endocytic and exocytic processes of wheat germ agglutinin by quantum dot-based single-particle tracking. Biomaterials 32, 7616–7624. https://doi.org/10.1016/j.biomaterials.2011.06.046

Liu, X.Z., Xu, X.M., Hu, R., Du, C., Zhang, S.X., McDonald, J.W., Dong, H.X., Wu, Y.J., Fan, G.S., Jacquin, M.F., Hsu, C.Y., Choi, D.W., 1997. Neuronal and Glial Apoptosis after Traumatic Spinal Cord Injury. J. Neurosci. 17, 5395–5406. https://doi.org/10.1523/JNEUROSCI.17-14-05395.1997

Lo, L., Anderson, D.J., 2011. A Cre-Dependent, Anterograde Transsynaptic Viral Tracer for Mapping Output Pathways of Genetically Marked Neurons. Neuron 72, 938–950. https://doi.org/10.1016/j.neuron.2011.12.002

Lord-Fontaine, S., Yang, F., Diep, Q., Dergham, P., Munzer, S., Tremblay, P., McKerracher, L., 2008. Local Inhibition of Rho Signaling by Cell-Permeable Recombinant Protein BA-210 Prevents Secondary Damage and Promotes Functional Recovery following Acute Spinal Cord Injury. J. Neurotrauma 25, 1309–1322. https://doi.org/10.1089/neu.2008.0613

Lü, H.-Z., Wang, Y.-X., Zou, J., Li, Y., Fu, S.-L., Jin, J.-Q., Hu, J.-G., Lu, P.-H., 2010. Differentiation of neural precursor cell-derived oligodendrocyte progenitor cells following transplantation into normal and injured spinal cords. Differentiation 80, 228–240. https://doi.org/10.1016/j.diff.2010.09.179

Lu, P., Ceto, S., Wang, Y., Graham, L., Wu, D., Kumamaru, H., Staufenberg, E., Tuszynski, M.H., 2017. Prolonged human neural stem cell maturation supports recovery in injured rodent CNS. J. Clin. Invest. 127, 3287–3299. https://doi.org/10.1172/JCI92955

Lu, P., Graham, L., Wang, Y., Wu, D., Tuszynski, M., 2014. Promotion of Survival and Differentiation of Neural Stem Cells with Fibrin and Growth Factor Cocktails after Severe Spinal Cord Injury. J. Vis. Exp. https://doi.org/10.3791/50641

Lu, P., Jones, L.L., Snyder, E.Y., Tuszynski, M.H., 2003. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp. Neurol. 181, 115–129. https://doi.org/10.1016/S0014-4886(03)00037-2

Lu, P., Wang, Y., Graham, L., McHale, K., Gao, M., Wu, D., Brock, J., Blesch, A., Rosenzweig, E.S., Havton, L.A., Zheng, B., Conner, J.M., Marsala, M., Tuszynski, M.H., 2012. Long-Distance Growth and Connectivity of Neural Stem Cells after Severe Spinal Cord Injury. Cell 150, 1264–1273. https://doi.org/10.1016/j.cell.2012.08.020

Ma, H., Morey, R., O'Neil, R.C., He, Y., Daughtry, B., Schultz, M.D., Hariharan, M., Nery, J.R., Castanon, R., Sabatini, K., Thiagarajan, R.D., Tachibana, M., Kang, E., Tippner-Hedges, R., Ahmed, R., Gutierrez, N.M., Van Dyken, C., Polat, A., Sugawara, A., Sparman, M., Gokhale, S., Amato, P., P.Wolf, D., Ecker, J.R., Laurent, L.C., Mitalipov, S., 2014. Abnormalities in human pluripotent cells due to reprogramming mechanisms. Nature 511, 177–183. https://doi.org/10.1038/nature13551

Manley, N.C., Priest, C.A., Denham, J., Wirth, E.D., Lebkowski, J.S., 2017. Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cells: Preclinical Efficacy and Safety in Cervical Spinal Cord Injury: hESC-Derived OPCs for Cervical Spinal Cord Injury. STEM CELLS Transl. Med. 6, 1917–1929. https://doi.org/10.1002/sctm.17-0065

Martino, G., Pluchino, S., 2006. The therapeutic potential of neural stem cells. Nat. Rev. Neurosci. 7, 395–406. https://doi.org/10.1038/nrn1908

Martirosyan, N.L., Patel, A.A., Carotenuto, A., Kalani, M.Y.S., Bohl, M.A., Preul, M.C., Theodore, N., 2017. The role of therapeutic hypothermia in the management of acute

spinal cord injury. Clin. Neurol. Neurosurg. 154, 79–88. https://doi.org/10.1016/j.clineuro.2017.01.002

Maskos, U., Kissa, K., Cloment, C.S., Brûlet, P., 2002. Retrograde trans-synaptic transfer of green fluorescent protein allows the genetic mapping of neuronal circuits in transgenic mice. Proc. Natl. Acad. Sci. 99, 10120–10125.

Matzke, M.A., Mette, M.F., Matzke, A.J.M., 2000. Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates, in: Matzke, M.A., Matzke, A.J.M. (Eds.), Plant Gene Silencing. Springer Netherlands, Dordrecht, pp. 281–295. https://doi.org/10.1007/978-94-011-4183-3_20

Maxwell, D.J., Belle, M.D., Cheunsuang, O., Stewart, A., Morris, R., 2007. Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn: Spinal interneurons. J. Physiol. 584, 521–533. https://doi.org/10.1113/jphysiol.2007.140996

Mayr, W., Krenn, M., Dimitrijevic, M.R., 2016. Epidural and transcutaneous spinal electrical stimulation for restoration of movement after incomplete and complete spinal cord injury: Curr. Opin. Neurol. 29, 721–726. https://doi.org/10.1097/WCO.0000000000000382

McCall, A.A., Miller, D.M., Yates, B.J., 2017. Descending Influences on Vestibulospinal and Vestibulosympathetic Reflexes. Front. Neurol. 8. https://doi.org/10.3389/fneur.2017.00112

McGovern, A.E., Davis-Poynter, N., Rakoczy, J., Phipps, S., Simmons, D.G., Mazzone, S.B., 2012. Anterograde neuronal circuit tracing using a genetically modified herpes simplex virus expressing EGFP. J. Neurosci. Methods 209, 158–167. https://doi.org/10.1016/j.jneumeth.2012.05.035

McKerracher, L., Anderson, K.D., 2013. Analysis of Recruitment and Outcomes in the Phase I/IIa Cethrin Clinical Trial for Acute Spinal Cord Injury. J. Neurotrauma 30, 1795–1804. https://doi.org/10.1089/neu.2013.2909

Means, E.D., Anderson, D.K., Waters, T.R., Kalaf, L., 1981. Effect of methylprednisolone in compression trauma to the feline spinal cord. J. Neurosurg. 55, 200–208.

Mestre, H., Ramirez, M., Garcia, E., MartiÃ±Ã^{3}n, S., Cruz, Y., Campos, M.G., Ibarra, A., 2015. Lewis, Fischer 344, and Sprague-Dawley Rats Display Differences in Lipid Peroxidation, Motor Recovery, and Rubrospinal Tract Preservation after Spinal Cord Injury. Front. Neurol. 6. https://doi.org/10.3389/fneur.2015.00108

Metz, G.A., Whishaw, I.Q., 2009. The Ladder Rung Walking Task: A Scoring System and its Practical Application. J. Vis. Exp. https://doi.org/10.3791/1204

Mizuguchi, H., Xu, Z., Ishii-Watabe, A., Uchida, E., Hayakawa, T., 2000. IRES-Dependent Second Gene Expression Is Significantly Lower Than Cap-Dependent First Gene Expression in a Bicistronic Vector. Mol. Ther. 1, 376–382. https://doi.org/10.1006/mthe.2000.0050

Montoya, C.P., Campbell-Hope, L.J., Pemberton, K.D., Dunnett, S.B., 1991. The “staircase test”: a measure of independent forelimb reaching and grasping abilities in rats. J. Neurosci. Methods 36, 219–228. https://doi.org/10.1016/0165-0270(91)90048-5

Morita, T., Sasaki, M., Kataoka-Sasaki, Y., Nakazaki, M., Nagahama, H., Oka, S., Oshigiri, T., Takebayashi, T., Yamashita, T., Kocsis, J.D., Honmou, O., 2016. Intravenous infusion of mesenchymal stem cells promotes functional recovery in a model of chronic spinal cord injury. Neuroscience 335, 221–231. https://doi.org/10.1016/j.neuroscience.2016.08.037

Morris, R., Whishaw, I.Q., 2016. A Proposal for a Rat Model of Spinal Cord Injury Featuring the Rubrospinal Tract and its Contributions to Locomotion and Skilled Hand Movement. Front. Neurosci. 10. https://doi.org/10.3389/fnins.2016.00005

Morrissey, T., Kleitman, N., Bunge, R., 1991. Isolation and functional characterization of Schwann cells derived from adult peripheral nerve. J. Neurosci. 11, 2433–2442. https://doi.org/10.1523/JNEUROSCI.11-08-02433.1991

Morshead, C.M., Reynolds, B.A., Craig, C.G., McBurney, M.W., Staines, W.A., Morassutti, D., Weiss, S., van der Kooy, D., 1994. Neural stem cells in the adult mammalian forebrain: A relatively quiescent subpopulation of subependymal cells. Neuron 13, 1071–1082. https://doi.org/10.1016/0896-6273(94)90046-9

Mothe, A.J., Kulbatski, I., Parr, A., Mohareb, M., Tator, C.H., 2008. Adult Spinal Cord Stem/Progenitor Cells Transplanted as Neurospheres Preferentially Differentiate into Oligodendrocytes in the Adult Rat Spinal Cord. Cell Transplant. 17, 735–751. https://doi.org/10.3727/096368908786516756

Mothe, A.J., Tator, C.H., 2013. Review of transplantation of neural stem/progenitor cells for spinal cord injury. Int. J. Dev. Neurosci. 31, 701–713. https://doi.org/10.1016/j.ijdevneu.2013.07.004

Muir, G.D., Webb, A.A., 2000. Assessment of behavioural recovery following spinal cord injury in rats: Behavioural assessment in spinal-injured rats. Eur. J. Neurosci. 12, 3079–3086. https://doi.org/10.1046/j.1460-9568.2000.00205.x

Munce, S.E., Wodchis, W.P., Guilcher, S.J., Couris, C.M., Verrier, M., Fung, K., Craven, B.C., Jaglal, S.B., 2013. Direct costs of adult traumatic spinal cord injury in Ontario. Spinal Cord 51, 64.

Nagata, Y., Burger, M.M., 1972. Wheat germ agglutinin isolation and crystallization. J. Biol. Chem. 247, 2248–2250.

Nagoshi, N., Khazaei, M., Ahlfors, J.-E., Ahuja, C.S., Nori, S., Wang, J., Shibata, S., Fehlings, M.G., 2018. Human Spinal Oligodendrogenic Neural Progenitor Cells Promote Functional Recovery After Spinal Cord Injury by Axonal Remyelination and Tissue Sparing: Oligodendrogenic NPCs for Spinal Cord Injury. STEM CELLS Transl. Med. 7, 806–818. https://doi.org/10.1002/sctm.17-0269

Nagoshi, N., Shibata, S., Hamanoue, M., Mabuchi, Y., Matsuzaki, Y., Toyama, Y., Nakamura, M., Okano, H., 2011. Schwann cell plasticity after spinal cord injury shown by neural crest lineage tracing. Glia 59, 771–784. https://doi.org/10.1002/glia.21150

Nakafuku, M., Nagao, M., Grande, A., Cancelliere, A., 2008. Revisiting neural stem cell identity. Proc. Natl. Acad. Sci. 105, 829–830. https://doi.org/10.1073/pnas.0711637105

Nakagawa, H., Ninomiya, T., Yamashita, T., Takada, M., 2015. Reorganization of corticospinal tract fibers after spinal cord injury in adult macaques. Sci. Rep. 5. https://doi.org/10.1038/srep11986

Nakhjavan-Shahraki, B., Yousefifard, M., Rahimi-Movaghar, V., Baikpour, M., Nasirinezhad, F., Safari, S., Yaseri, M., Moghadas Jafari, A., Ghelichkhani, P., Tafakhori, A., Hosseini, M., 2018. Transplantation of olfactory ensheathing cells on functional recovery and neuropathic pain after spinal cord injury; systematic review and meta-analysis. Sci. Rep. 8. https://doi.org/10.1038/s41598-017-18754-4

Nathan, P.W., Smith, M.C., 1982. The rubrospinal and central tegmental tracts in man. Brain 105, 223–269. https://doi.org/10.1093/brain/105.2.223

Neubauer, V., Helting, T.B., 1981. Struture of Tetanus Toxin: The arrangement of papain digestion products within the heavy chain-light chain framework of extracellular toxin. Biochim. Biophys. Acta 668, 141–148.

Niederöst, B., Oertle, T., Fritsche, J., McKinney, R.A., Bandtlow, C.E., 2002. Nogo-A and Myelin-Associated Glycoprotein Mediate Neurite Growth Inhibition by Antagonistic Regulation of RhoA and Rac1. J. Neurosci. 22, 10368–10376. https://doi.org/10.1523/JNEUROSCI.22-23-10368.2002

Nishiyama, J., Mikuni, T., Yasuda, R., 2017. Virus-Mediated Genome Editing via Homology-Directed Repair in Mitotic and Postmitotic Cells in Mammalian Brain. Neuron 96, 755-768.e5. https://doi.org/10.1016/j.neuron.2017.10.004

Nistor, G.I., Totoiu, M.O., Haque, N., Carpenter, M.K., Keirstead, H.S., 2005. Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia 49, 385–396. https://doi.org/10.1002/glia.20127

Norgren, R.B., N. Lehman, M., 1998. Herpes simplex virus as a transneuronal tracer. Neurosci. Biobehav. Rev. 22, 695–708. https://doi.org/10.1016/S0149-7634(98)00008-6

Nori, S., Khazaei, M., Ahuja, C.S., Yokota, K., Ahlfors, J.-E., Liu, Y., Wang, J., Shibata, S., Chio, J., Hettiaratchi, M.H., Führmann, T., Shoichet, M.S., Fehlings, M.G., 2018. Human Oligodendrogenic Neural Progenitor Cells Delivered with Chondroitinase ABC Facilitate Functional Repair of Chronic Spinal Cord Injury. Stem Cell Rep. 11, 1433–1448. https://doi.org/10.1016/j.stemcr.2018.10.017

Norrman, K., Fischer, Y., Bonnamy, B., Wolfhagen Sand, F., Ravassard, P., Semb, H., 2010. Quantitative Comparison of Constitutive Promoters in Human ES cells. PLoS ONE 5, e12413. https://doi.org/10.1371/journal.pone.0012413

Nudo, R.J., Masterton, R.B., 1988. Descending pathways to the spinal cord: A comparative study of 22 mammals. J. Comp. Neurol. 277, 53–79. https://doi.org/10.1002/cne.902770105

Oh, J.S., Kim, K.N., An, S.S., Pennant, W.A., Kim, H.J., Gwak, S.J., Yoon, D.H., Lim, M.H., Choi, B.H., Ha, Y., 2011. Cotransplantation of mouse neural stem cells (mNSCs) with adipose tissue-derived mesenchymal stem cells improves mNSC survival in a rat spinal cord injury model. Cell Transpl. 20, 837–49. https://doi.org/10.3727/096368910x539083

Ohmori, T., Nagao, Y., Mizukami, H., Sakata, A., Muramatsu, S., Ozawa, K., Tominaga, S., Hanazono, Y., Nishimura, S., Nureki, O., Sakata, Y., 2017. CRISPR/Cas9-mediated genome editing via postnatal administration of AAV vector cures haemophilia B mice. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-04625-5

Oikari, L.E., Okolicsanyi, R.K., Griffiths, L.R., Haupt, L.M., 2016. Data defining markers of human neural stem cell lineage potential. Data Brief 7, 206–215. https://doi.org/10.1016/j.dib.2016.02.030

Okada, S., Ishii, K., Yamane, J., Iwanami, A., Ikegami, T., Katoh, H., Iwamoto, Y., Nakamura, M., Miyoshi, H., Okano, H.J., Contag, C.H., Toyama, Y., Okano, H., 2005. In vivo imaging of engrafted neural stem cells: its application in evaluating the optimal timing of transplantation for spinal cord injury. FASEB J. 19, 1839–1841. https://doi.org/10.1096/fj.05-4082fje

Okano, H., Kawahara, H., Toriya, M., Nakao, K., Shibata, S., Imai, T., 2005. Function of RNA-binding protein Musashi-1 in stem cells. Exp. Cell Res. 306, 349–356. https://doi.org/10.1016/j.yexcr.2005.02.021

Onifer, S.M., Rodríguez, J.F., Santiago, D.I., Benitez, J.C., Kim, D.T., Brunschwig, J.-P.R., Pacheco, J.T., Perrone, J.V., Llorente, O., Hesse, D.H., Martinez-Arizala, A., 1997. Cervical spinal cord injury in the adult rat: assessment of forelimb dysfunction. Restor Neurol Neurosci 11, 211–223.

Orth, P., Schnappinger, D., Hillen, W., Saenger, W., Hinrichs, W., 2000. Structural basis of gene regulation by the tetracycline inducible Tet repressor–operator system. Nat. Struct. Biol. 7, 5.

Oztas, E., 2003. Neuronal tracing. Neuroanatomy 2, 2–5.

Park, E., Velumian, A.A., Fehlings, M.G., 2004. The Role of Excitotoxicity in Secondary Mechanisms of Spinal Cord Injury: A Review with an Emphasis on the Implications for White Matter Degeneration. J. Neurotrauma 21, 754–774. https://doi.org/10.1089/0897715041269641

Parr, A.M., Kulbatski, I., Tator, C.H., 2007. Transplantation of Adult Rat Spinal Cord Stem/Progenitor Cells for Spinal Cord Injury. J. Neurotrauma 24, 835–845. https://doi.org/10.1089/neu.2006.3771

Parr, A.M., Kulbatski, I., Zahir, T., Wang, X., Yue, C., Keating, A., Tator, C.H., 2008. Transplanted adult spinal cord–derived neural stem/progenitor cells promote early

functional recovery after rat spinal cord injury. Neuroscience 155, 760–770. https://doi.org/10.1016/j.neuroscience.2008.05.042

Paxinos, G., Watson, C., 2004. The Rat Brain in Stereotaxic Coordinates - The New COronal Set, 5th ed. Elsevier.

Pearse, D., Bastidas, J., Izabel, S., Ghosh, M., 2018. Schwann Cell Transplantation Subdues the Pro-Inflammatory Innate Immune Cell Response after Spinal Cord Injury. Int. J. Mol. Sci. 19, 2550. https://doi.org/10.3390/ijms19092550

Pfaff, N., Lachmann, N., Ackermann, M., Kohlscheen, S., Brendel, C., Maetzig, T., Niemann, H., Antoniou, M.N., Grez, M., Schambach, A., Cantz, T., Moritz, T., 2013. A ubiquitous chromatin opening element prevents transgene silencing in pluripotent stem cells and their differentiated progeny. STEM CELLS 31, 488–499. https://doi.org/10.1002/stem.1316

Pickett, G.E., Campos-Benitez, M., Keller, J.L., Duggal, N., 2006. Epidemiology of traumatic spinal cord injury in Canada. Spine 31, 799–805.

Piltti, K.M., Avakian, S.N., Funes, G.M., Hu, A., Uchida, N., Anderson, A.J., Cummings, B.J., 2015. Transplantation dose alters the dynamics of human neural stem cell engraftment, proliferation and migration after spinal cord injury. Stem Cell Res. 15, 341–353. https://doi.org/10.1016/j.scr.2015.07.001

Piltti, K.M., Funes, G.M., Avakian, S.N., Salibian, A.A., Huang, K.I., Carta, K., Kamei, N., Flanagan, L.A., Monuki, E.S., Uchida, N., Cummings, B.J., Anderson, A.J., 2017. Increasing Human Neural Stem Cell Transplantation Dose Alters Oligodendroglial and Neuronal Differentiation after Spinal Cord Injury. Stem Cell Rep. 8, 1534–1548. https://doi.org/10.1016/j.stemcr.2017.04.009

Piltti, K.M., Salazar, D.L., Uchida, N., Cummings, B.J., Anderson, A.J., 2013. Safety of Epicenter Versus Intact Parenchyma as a Transplantation Site for Human Neural Stem Cells for Spinal Cord Injury Therapy. STEM CELLS Transl. Med. 2, 204–216. https://doi.org/10.5966/sctm.2012-0110

Plemel, J.R., Chojnacki, A., Sparling, J.S., Liu, J., Plunet, W., Duncan, G.J., Park, S.E., Weiss, S., Tetzlaff, W., 2011. Platelet-derived growth factor-responsive neural precursors give rise to myelinating oligodendrocytes after transplantation into the spinal cords of contused rats and dysmyelinated mice. Glia 59, 1891–1910. https://doi.org/10.1002/glia.21232

Polderman, K.H., 2009. Mechanisms of action, physiological effects, and complications of hypothermia: Crit. Care Med. 37, S186–S202. https://doi.org/10.1097/CCM.0b013e3181aa5241

Pomeshchik, Y., Puttonen, K.A., Kidin, I., Ruponen, M., Lehtonen, S., Malm, T., Åkesson, E., Hovatta, O., Koistinaho, J., 2015. Transplanted Human Induced Pluripotent Stem Cell-Derived Neural Progenitor Cells Do Not Promote Functional Recovery of

Pharmacologically Immunosuppressed Mice with Contusion Spinal Cord Injury. Cell Transplant. 24, 1799–1812. https://doi.org/10.3727/096368914X684079

Priest, C.A., Manley, N.C., Denham, J., Wirth, E.D., Lebkowski, J.S., 2015. Preclinical safety of human embryonic stem cell-derived oligodendrocyte progenitors supporting clinical trials in spinal cord injury. Regen. Med. 10, 939–958. https://doi.org/10.2217/rme.15.57

Prinjha, R., Moore, S.E., Vinson, M., Blake, S., Morrow, R., Christie, G., Michalovich, D., Simmons, D.L., Walsh, F.S., 2000. Inhibitor of neurite outgrowth in humans. Nat. Commun. 403, 383–384.

Qian, X., Shen, Q., Goderie, S.K., He, W., Capela, A., Davis, A.A., Temple, S., 2000. Timing of CNS Cell Generation: A Programmed Sequence of Neuron and Glial Cell Production from Isolated Murine Cortical Stem Cells. Neuron 28, 69–80.

Qin, J.Y., Zhang, L., Clift, K.L., Hulur, I., Xiang, A.P., Ren, B.-Z., Lahn, B.T., 2010. Systematic Comparison of Constitutive Promoters and the Doxycycline-Inducible Promoter. PLoS ONE 5, e10611. https://doi.org/10.1371/journal.pone.0010611

Quertainmont, R., Cantinieaux, D., Botman, O., Sid, S., Schoenen, J., Franzen, R., 2012. Mesenchymal Stem Cell Graft Improves Recovery after Spinal Cord Injury in Adult Rats through Neurotrophic and Pro-Angiogenic Actions. PLoS ONE 7, e39500. https://doi.org/10.1371/journal.pone.0039500

Redondo-Castro, E., Torres-Espín, A., García-Alías, G., Navarro, X., 2013. Quantitative assessment of locomotion and interlimb coordination in rats after different spinal cord injuries. J. Neurosci. Methods 213, 165–178. https://doi.org/10.1016/j.jneumeth.2012.12.024

Reeber, S.L., Gebre, S.A., Filatova, N., Sillitoe, R.V., 2011. Revealing Neural Circuit Topography in Multi-Color. J. Vis. Exp. https://doi.org/10.3791/3371

Reiner, A., Veenman, C.L., Medina, L., Jiao, Y., Del Mar, N., Honig, M.G., 2000. Pathway tracing using biotinylated dextran amines. J. Neurosci. Methods 103, 23–37. https://doi.org/10.1016/S0165-0270(00)00293-4

Rejc, E., Angeli, C.A., Atkinson, D., Harkema, S.J., 2017a. Motor recovery after activity-based training with spinal cord epidural stimulation in a chronic motor complete paraplegic. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-14003-w

Rejc, E., Angeli, C.A., Bryant, N., Harkema, S.J., 2017b. Effects of Stand and Step Training with Epidural Stimulation on Motor Function for Standing in Chronic Complete Paraplegics. J. Neurotrauma 34, 1787–1802. https://doi.org/10.1089/neu.2016.4516

Reynolds, B., Weiss, S., 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255, 1707–1710. https://doi.org/10.1126/science.1553558

Richards, L.J., Kilpatrick, T.J., Bartlett, P.F., 1992. De novo generation of neuronal cells from the adult mouse brain. Proc. Natl. Acad. Sci. 89, 8591–8595. https://doi.org/10.1073/pnas.89.18.8591

Rietze, R.L., Reynolds, B.A., 2006. Neural Stem Cell Isolation and Characterization, in: Methods in Enzymology. Elsevier, pp. 3–23. https://doi.org/10.1016/S0076-6879(06)19001-1

Rivlin, A.S. and Tator, C.H., 1978. Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat. Surgical neurology, 10(1), pp.38-43.

Robinson, J., Lu, P., 2017. Optimization of trophic support for neural stem cell grafts in sites of spinal cord injury. Exp. Neurol. 291, 87–97. https://doi.org/10.1016/j.expneurol.2017.02.007

Roet, K.C.D., Verhaagen, J., 2014. Understanding the neural repair-promoting properties of olfactory ensheathing cells. Exp. Neurol. 261, 594–609. https://doi.org/10.1016/j.expneurol.2014.05.007

Rosenblum, S., Smith, T.N., Wang, N., Chua, J.Y., Westbroek, E., Wang, K., Guzman, R., 2015. BDNF Pretreatment of Human Embryonic-Derived Neural Stem Cells Improves Cell Survival and Functional Recovery after Transplantation in Hypoxic–Ischemic Stroke. Cell Transplant. 24, 2449–2461. https://doi.org/10.3727/096368914X679354

Rosich, K., Hanna, B.F., Ibrahim, R.K., Hellenbrand, D.J., Hanna, A., 2017. The Effects of Glial Cell Line-Derived Neurotrophic Factor after Spinal Cord Injury. J. Neurotrauma 34, 3311–3325. https://doi.org/10.1089/neu.2017.5175

Ruan, J., Li, H., Xu, K., Wu, T., Wei, J., Zhou, R., Liu, Z., Mu, Y., Yang, S., Ouyang, H., Yanru Chen-Tsai, R., Li, K., 2015. Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. Sci. Rep. 5. https://doi.org/10.1038/srep14253

Ruda, M.A., Coulter, J.D., 1982. Axonal and transneuronal transport of wheat germ agglutinin demonstrated by immunocytochemistry. Brain Res. 249, 237–246.

Ryken, T.C., Hurlbert, R.J., Hadley, M.N., Aarabi, B., Dhall, S.S., Gelb, D.E., Rozzelle, C.J., Theodore, N., Walters, B.C., 2013. The Acute Cardiopulmonary Management of Patients With Cervical Spinal Cord Injuries: Neurosurgery 72, 84–92. https://doi.org/10.1227/NEU.0b013e318276ee16

Sabelstrom, H., Stenudd, M., Reu, P., Dias, D.O., Elfineh, M., Zdunek, S., Damberg, P., Goritz, C., Frisen, J., 2013. Resident Neural Stem Cells Restrict Tissue Damage and Neuronal Loss After Spinal Cord Injury in Mice. Science 342, 637–640. https://doi.org/10.1126/science.1242576

Sadowsky, C.L., Hammond, E.R., Strohl, A.B., Commean, P.K., Eby, S.A., Damiano, D.L., Wingert, J.R., Bae, K.T., McDonald, J.W., 2013. Lower extremity functional electrical stimulation cycling promotes physical and functional recovery in chronic spinal cord

injury. J. Spinal Cord Med. 36, 623–631. https://doi.org/10.1179/2045772313Y.0000000101

Sahni, V., Kessler, J.A., 2010. Stem cell therapies for spinal cord injury. Nat. Rev. Neurol. 6, 363–372. https://doi.org/10.1038/nrneurol.2010.73

Salazar, D.L., Uchida, N., Hamers, F.P.T., Cummings, B.J., Anderson, A.J., 2010. Human Neural Stem Cells Differentiate and Promote Locomotor Recovery in an Early Chronic Spinal coRd Injury NOD-scid Mouse Model. PLoS ONE 5, e12272. https://doi.org/10.1371/journal.pone.0012272

Salewski, R.P., Mitchell, R.A., Shen, C., Fehlings, M.G., 2015. Transplantation of Neural Stem Cells Clonally Derived from Embryonic Stem Cells Promotes Recovery After Murine Spinal Cord Injury. Stem Cells Dev. 24, 36–50. https://doi.org/10.1089/scd.2014.0096

Satkunendrarajah, K., Nassiri, F., Karadimas, S.K., Lip, A., Yao, G., Fehlings, M.G., 2016. Riluzole promotes motor and respiratory recovery associated with enhanced neuronal survival and function following high cervical spinal hemisection. Exp. Neurol. 276, 59–71. https://doi.org/10.1016/j.expneurol.2015.09.011

Sayer, F.T., Kronvall, E., Nilsson, O.G., 2006. Methylprednisolone treatment in acute spinal cord injury: the myth challenged through a structured analysis of published literature. Spine J. 6, 335–343. https://doi.org/10.1016/j.spinee.2005.11.001

Schnell, L., Schwab, M.E., 1990. Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature 343, 269–272. https://doi.org/10.1038/343269a0

Schnütgen, F., Doerflinger, N., Calléja, C., Wendling, O., Chambon, P., Ghyselinck, N.B., 2003. A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse. Nat. Biotechnol. 21, 562–565. https://doi.org/10.1038/nbt811

Scholle, H.Ch., Schumann, N.P., Biedermann, F., Stegeman, D.F., Graßme, R., Roeleveld, K., Schilling, N., Fischer, M.S., 2001. Spatiotemporal surface EMG characteristics from rat triceps brachii muscle during treadmill locomotion indicate selective recruitment of functionally distinct muscle regions. Exp. Brain Res. 138, 26–36. https://doi.org/10.1007/s002210100685

Scholtes, F., Theunissen, E., Phan-Ba, R., Adriaensens, P., Brook, G., Franzen, R., Gelan, J., Schoenen, J., Martin, D., 2011. Post-mortem assessment of rat spinal cord injury and white matter sparing using inversion recovery-supported proton density magnetic resonance imaging. Spinal Cord 49, 345–351. https://doi.org/10.1038/sc.2010.129

Schroeder, G.D., Kwon, B.K., Eck, J.C., Savage, J.W., Hsu, W.K., Patel, A.A., 2014. Survey of Cervical Spine Research Society Members on the Use of High-Dose Steroids for Acute Spinal Cord Injuries: Spine 39, 971–977. https://doi.org/10.1097/BRS.0000000000000297

Schwab, M.E., Thoenen, H., 1976. Electron microscopic evidence for a transsynaptic migration of tetanus toxin in spinal cord motoneurons: An autoradiographic and morphometric study. Brain Res. 105, 213–227. https://doi.org/10.1016/0006-8993(76)90422-4

Schweigreiter, R., Bandtlow, C.E., 2006. Nogo in the Injured Spinal Cord. J. Neurotrauma 23, 384–396. https://doi.org/10.1089/neu.2006.23.384

Scott, C.T., Magnus, D., 2014. Wrongful Termination: Lessons From the Geron Clinical Trial: Lessons From the Geron Trial. STEM CELLS Transl. Med. 3, 1398–1401. https://doi.org/10.5966/sctm.2014-0147

Sekhon, L.H., Fehlings, M.G., 2001. Epidemiology, demographics, and pathophysiology of acute spinal cord injury. Spine 26, S2–S12.

Semple, B.D., Blomgren, K., Gimlin, K., Ferriero, D.M., Noble-Haeusslein, L.J., 2013. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog. Neurobiol. 106–107, 1–16. https://doi.org/10.1016/j.pneurobio.2013.04.001

Senís, E., Fatouros, C., Große, S., Wiedtke, E., Niopek, D., Mueller, A.-K., Börner, K., Grimm, D., 2014. CRISPR/Cas9-mediated genome engineering: An adeno-associated viral (AAV) vector toolbox. Biotechnol. J. 9, 1402–1412. https://doi.org/10.1002/biot.201400046

Serradj, N., Agger, S.F., Hollis, E.R., 2017. Corticospinal circuit plasticity in motor rehabilitation from spinal cord injury. Neurosci. Lett. 652, 94–104. https://doi.org/10.1016/j.neulet.2016.12.003

Serrano-Martin, X., Payares, G., Mendoza-Leon, A., 2006. Glibenclamide, a Blocker of K+ATP Channels, Shows Antileishmanial Activity in Experimental Murine Cutaneous Leishmaniasis. Antimicrob. Agents Chemother. 50, 4214–4216. https://doi.org/10.1128/AAC.00617-06

Sharif-Alhoseini, M., Khormali, M., Rezaei, M., Safdarian, M., Hajighadery, A., Khalatbari, M.M., Safdarian, M., Meknatkhah, S., Rezvan, M., Chalangari, M., Derakhshan, P., Rahimi-Movaghar, V., 2017. Animal models of spinal cord injury: a systematic review. Spinal Cord 55, 714–721. https://doi.org/10.1038/sc.2016.187

Sharp, J., Frame, J., Siegenthaler, M., Nistor, G., Keirstead, H.S., 2009. Human embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cell Transplants Improve Recovery after Cervical Spinal Cord Injury. Stem Cells N/A-N/A. https://doi.org/10.1002/stem.245

Shaw, P.J., Ince, P.G., 1997. Glutamate, excitotoxicity and amyotrophic lateral sclerosis. J. Neurol. 244, S3–S14. https://doi.org/10.1007/BF03160574

Simard, J.M., Chen, M., Tarasov, K.V., Bhatta, S., Ivanova, S., Melnitchenko, L., Tsymbalyuk, N., West, G.A., Gerzanich, V., 2006. Newly expressed SUR1-regulated NCCa-ATP channel mediates cerebral edema after ischemic stroke. Nat. Med. 12, 433–440. https://doi.org/10.1038/nm1390

Simard, J.M., Tsymbalyuk, O., Ivanov, A., Ivanova, S., Bhatta, S., Geng, Z., Woo, S.K., Gerzanich, V., 2007. Endothelial sulfonylurea receptor 1–regulated NCCa-ATP channels mediate progressive hemorrhagic necrosis following spinal cord injury. J. Clin. Invest. 117, 2105–2113. https://doi.org/10.1172/JCI32041

Singh, A., Tetreault, L., Kalsi-Ryan, S., Nouri, A., Fehlings, M.G., 2014. Global prevalence and incidence of traumatic spinal cord injury. Clin. Epidemiol. 309. https://doi.org/10.2147/CLEP.S68889

Sipp, D., Robey, P.G., Turner, L., 2018. Clear up this stem-cell mess. Nature 561, 455–457. https://doi.org/10.1038/d41586-018-06756-9

Smith, A.C., Weber, K.A., O'Dell, D.R., Parrish, T.B., Wasielewski, M., Elliott, J.M., 2018. Lateral Corticospinal Tract Damage Correlates With Motor Output in Incomplete Spinal Cord Injury. Arch. Phys. Med. Rehabil. 99, 660–666. https://doi.org/10.1016/j.apmr.2017.10.002

Smith, B.N., Banfield, B.W., Smeraski, C.A., Wilcox, C.L., Dudek, F.E., Enquist, L.W., Pickard, G.E., 2000. Pseudorabies virus expressing enhanced green fluorescent protein: A tool for in vitro electrophysiological analysis of transsynaptically labeled neurons in identified central nervous system circuits. Proc. Natl. Acad. Sci. 97, 9264–9269. https://doi.org/10.1073/pnas.97.16.9264

Song, S.S., Lyden, P.D., 2012. Overview of Therapeutic Hypothermia. Curr. Treat. Options Neurol. 14, 541–548. https://doi.org/10.1007/s11940-012-0201-x

Spinal Cord Injury Facts and Figures at a Glance, 2014. . J. Spinal Cord Med. 37, 355–356. https://doi.org/10.1179/1079026814Z.000000000260

Spinal Cord Injury Facts and Figures at a Glance, 2012. . J. Spinal Cord Med. 35, 197–198. https://doi.org/10.1179/1079026812Z.00000000063

Stenudd, M., Sabelström, H., Frisén, J., 2015. Role of Endogenous Neural Stem Cells in Spinal Cord Injury and Repair. JAMA Neurol. 72, 235. https://doi.org/10.1001/jamaneurol.2014.2927

Stewart, A.N., Kendziorski, G., Deak, Z.M., Brown, D.J., Fini, M.N., Copely, K.L., Rossignol, J., Dunbar, G.L., 2017. Co-transplantation of mesenchymal and neural stem cells and overexpressing stromal-derived factor-1 for treating spinal cord injury. Brain Res 1672, 91–105. https://doi.org/10.1016/j.brainres.2017.07.005

Stirling, D.P., 2004. Minocycline Treatment Reduces Delayed Oligodendrocyte Death, Attenuates Axonal Dieback, and Improves Functional Outcome after Spinal Cord Injury. J. Neurosci. 24, 2182–2190. https://doi.org/10.1523/JNEUROSCI.5275-03.2004

Sugita, M., 2005. Genetic Tracing Shows Segregation of Taste Neuronal Circuitries for Bitter and Sweet. Science 309, 781–785. https://doi.org/10.1126/science.1110787

Sun, L., Tang, Y., Yan, K., Yu, J., Zou, Y., Xu, W., Xiao, K., Zhang, Z., Li, W., Wu, B., Hu, Z., Chen, K., Fu, Z.F., Dai, J., Cao, G., 2019. Differences in neurotropism and neurotoxicity among retrograde viral tracers. Mol. Neurodegener. 14. https://doi.org/10.1186/s13024-019-0308-6

Sun, T., Wang, X.-J., Xie, S.-S., Zhang, D.-L., Wang, X.-P., Li, B.-Q., Ma, W., Xin, H., 2011. A comparison of proliferative capacity and passaging potential between neural stem and progenitor cells in adherent and neurosphere cultures. Int. J. Dev. Neurosci. 29, 723–731. https://doi.org/10.1016/j.ijdevneu.2011.05.012

Suzuki, H., Ahuja, C.S., Salewski, R.P., Li, L., Satkunendrarajah, K., Nagoshi, N., Shibata, S., Fehlings, M.G., 2017. Neural stem cell mediated recovery is enhanced by Chondroitinase ABC pretreatment in chronic cervical spinal cord injury. PLOS ONE 12, e0182339. https://doi.org/10.1371/journal.pone.0182339

Swiech, L., Heidenreich, M., Banerjee, A., Habib, N., Li, Y., Trombetta, J., Sur, M., Zhang, F., 2015. In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nat. Biotechnol. 33, 102–106. https://doi.org/10.1038/nbt.3055

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. https://doi.org/10.1016/j.cell.2007.11.019

Takahashi, K., Yamanaka, S., 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126, 663–676. https://doi.org/10.1016/j.cell.2006.07.024

Takano, M., Kawabata, S., Shibata, S., Yasuda, A., Nori, S., Tsuji, O., Nagoshi, N., Iwanami, A., Ebise, H., Horiuchi, K., Okano, H., Nakamura, M., 2017. Enhanced Functional Recovery from Spinal Cord Injury in Aged Mice after Stem Cell Transplantation through HGF Induction. Stem Cell Rep. 8, 509–518. https://doi.org/10.1016/j.stemcr.2017.01.013

Takeuchi, Y., Matsumoto, Y., Miki, T., Yokoyama, T., Warita, K., Wang, Z.-Y., Ueno, T., Yakura, T., Fujita, M., 2009. Anterograde synaptic transport of neuronal tracer enzyme (WGA-HRP): further studies with Rab3A-siRNA in rats. Biomed. Res. - India 20, 149. https://doi.org/10.4103/0970-938X.54832

Tarasenko, Y.I., Gao, J., Nie, L., Johnson, K.M., Grady, J.J., Hulsebosch, C.E., McAdoo, D.J., Wu, P., 2007. Human fetal neural stem cells grafted into contusion-injured rat spinal cords improve behavior. J. Neurosci. Res. 85, 47–57. https://doi.org/10.1002/jnr.21098

Tashiro, S., Nishimura, S., Iwai, H., Sugai, K., Zhang, L., Shinozaki, M., Iwanami, A., Toyama, Y., Liu, M., Okano, H., Nakamura, M., 2016. Functional Recovery from Neural Stem/Progenitor Cell Transplantation Combined with Treadmill Training in Mice with Chronic Spinal Cord Injury. Sci Rep 6, 30898. https://doi.org/10.1038/srep30898

Tator, C.H., 2006. Review of Treatment Trials in Human Spinal Cord Injury. Neurosurgery 59, 957–987. https://doi.org/10.1227/01.NEU.0000245591.16087.89

Tator, C.H., Fehlings, M.G., 1991. Review of the secondary injury theroy of acute spinal cord trauma with emphasis on vascular mechanisms. J. Neurosurg. 75, 15–26. https://doi.org/10.3171/jns.1991.75.1.0015

Teng, Y.D., Choi, H., Onario, R.C., Zhu, S., Desilets, F.C., Lan, S., Woodard, E.J., Snyder, E.Y., Eichler, M.E., Friedlander, R.M., 2004. Minocycline inhibits contusion-triggered mitochondrial cytochrome c release and mitigates functional deficits after spinal cord injury. Proc. Natl. Acad. Sci. 101, 3071–3076. https://doi.org/10.1073/pnas.0306239101

Teng, Y.D., Lavik, E.B., Qu, X., Park, K.I., Ourednik, J., Zurakowski, D., Langer, R., Snyder, E.Y., 2002. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc. Natl. Acad. Sci. 99, 3024–3029. https://doi.org/10.1073/pnas.052678899

Terson de Paleville, D., McKay, W., Aslan, S., Folz, R., Sayenko, D., Ovechkin, A., 2013. Locomotor step training with body weight support improves respiratory motor function in individuals with chronic spinal cord injury. Respir. Physiol. Neurobiol. 189, 491–497. https://doi.org/10.1016/j.resp.2013.08.018

Tetzlaff, W., Okon, E.B., Karimi-Abdolrezaee, S., Hill, C.E., Sparling, J.S., Plemel, J.R., Plunet, W.T., Tsai, E.C., Baptiste, D., Smithson, L.J., Kawaja, M.D., Fehlings, M.G., Kwon, B.K., 2011. A Systematic Review of Cellular Transplantation Therapies for Spinal Cord Injury. J. Neurotrauma 28, 1611–1682. https://doi.org/10.1089/neu.2009.1177

Tolar, J., Le Blanc, K., Keating, A., Blazar, B.R., 2010. Concise Review: Hitting the Right Spot with Mesenchymal Stromal Cells. STEM CELLS 28, 1446–1455. https://doi.org/10.1002/stem.459

Triolo, R.J., Bailey, S.N., Miller, M.E., Lombardo, L.M., Audu, M.L., 2013. Effects of Stimulating Hip and Trunk Muscles on Seated Stability, Posture, and Reach After Spinal Cord Injury. Arch. Phys. Med. Rehabil. 94, 1766–1775. https://doi.org/10.1016/j.apmr.2013.02.023

Tsutsumi, S., Ueta, T., Shiba, K., Yamamoto, S., Takagishi, K., 2006. Effects of the second national acute spinal cord injury study of high-dose methylprednisolone therapy on acute cervical spinal cord injury–results in spinal injuries center. Spine 31, 2992–2996.

Tu, T.-W., Kim, J.H., Yin, F.Q., Jakeman, L.B., Song, S.-K., 2013. The impact of myelination on axon sparing and locomotor function recovery in spinal cord injury assessed using diffusion tensor imaging: *IN VIVO* DTI OF DYSMYELINATED WHITE MATTER INTEGRITY IN SCI. NMR Biomed. 26, 1484–1495. https://doi.org/10.1002/nbm.2981

Ulndreaj, A., Badner, A., Fehlings, M.G., 2017. Promising neuroprotective strategies for traumatic spinal cord injury with a focus on the differential effects among anatomical levels of injury. F1000 Res. 1907, 1–13.

Vale, F.L., Burns, J., Jackson, A.B., Hadley, M.N., 1997. Combined medical and surgical treatment after acute spinal cord injury: results of a prospective pilot study to assess the

merits of aggressive medical resuscitation and blood pressure management. J. Neurosurg. 87, 239–246.

van der Sanden, B., Dhobb, M., Berger, F., Wion, D., 2010. Optimizing stem cell culture. J. Cell. Biochem. 111, 801–807. https://doi.org/10.1002/jcb.22847

van der Want, J.J.L., Klooster, J., Nunes Cardozo, B., de Weerd, H., Liem, R.S.B., 1997. Tract-tracing in the nervous system of vertebrates using horseradish peroxidase and its conjugates: tracers, chromogens and stabilization for light and electron microscopy. Brain Res. Protoc. 1, 269–279. https://doi.org/10.1016/S1385-299X(96)00042-6

Vaquero, J., Zurita, M., Rico, M.A., Bonilla, C., Aguayo, C., Fernández, C., Tapiador, N., Sevilla, M., Morejón, C., Montilla, J., Martínez, F., Marín, E., Bustamante, S., Vázquez, D., Carballido, J., Rodríguez, A., Martínez, P., García, C., Ovejero, M., Fernández, M.V., 2017. Repeated subarachnoid administrations of autologous mesenchymal stromal cells supported in autologous plasma improve quality of life in patients suffering incomplete spinal cord injury. Cytotherapy 19, 349–359. https://doi.org/10.1016/j.jcyt.2016.12.002

Vaquero, J., Zurita, M., Rico, M.A., Bonilla, C., Aguayo, C., Montilla, J., Bustamante, S., Carballido, J., Marin, E., Martinez, F., Parajon, A., Fernandez, C., Reina, L.D., 2016. An approach to personalized cell therapy in chronic complete paraplegia: The Puerta de Hierro phase I/II clinical trial. Cytotherapy 18, 1025–1036. https://doi.org/10.1016/j.jcyt.2016.05.003

Vawda, R., Badner, A., Hong, J., Mikhail, M., Lakhani, A., Dragas, R., Xhima, K., Barretto, T., Librach, C.L., Fehlings, M.G., 2019. Early Intravenous Infusion of Mesenchymal Stromal Cells Exerts a Tissue Source Age-Dependent Beneficial Effect on Neurovascular Integrity and Neurobehavioral Recovery After Traumatic Cervical Spinal Cord Injury. STEM CELLS Transl. Med. https://doi.org/10.1002/sctm.18-0192

Vismara, I., Papa, S., Rossi, F., Forloni, G., Veglianese, P., 2017. Current Options for Cell Therapy in Spinal Cord Injury. Trends Mol. Med. 23, 831–849. https://doi.org/10.1016/j.molmed.2017.07.005

von Leden, R.E., Khayrullina, G., Moritz, K.E., Byrnes, K.R., 2017. Age exacerbates microglial activation, oxidative stress, inflammatory and NOX2 gene expression, and delays functional recovery in a middle-aged rodent model of spinal cord injury. J. Neuroinflammation 14. https://doi.org/10.1186/s12974-017-0933-3

Wagner, F.B., Mignardot, J.-B., Le Goff-Mignardot, C.G., Demesmaeker, R., Komi, S., Capogrosso, M., Rowald, A., Seáñez, I., Caban, M., Pirondini, E., Vat, M., McCracken, L.A., Heimgartner, R., Fodor, I., Watrin, A., Seguin, P., Paoles, E., Van Den Keybus, K., Eberle, G., Schurch, B., Pralong, E., Becce, F., Prior, J., Buse, N., Buschman, R., Neufeld, E., Kuster, N., Carda, S., von Zitzewitz, J., Delattre, V., Denison, T., Lambert, H., Minassian, K., Bloch, J., Courtine, G., 2018. Targeted neurotechnology restores walking in humans with spinal cord injury. Nature 563, 65–71. https://doi.org/10.1038/s41586-018-0649-2

Wall, N.R., Wickersham, I.R., Cetin, A., De La Parra, M., Callaway, E.M., 2010. Monosynaptic circuit tracing in vivo through Cre-dependent targeting and complementation of modified rabies virus. Proc. Natl. Acad. Sci. 107, 21848–21853. https://doi.org/10.1073/pnas.1011756107

Walters, B.C., Hadley, M.N., Hurlbert, R.J., Aarabi, B., Dhall, S.S., Gelb, D.E., Harrigan, M.R., Rozelle, C.J., Ryken, T.C., Theodore, N., 2013. Guidelines for the Management of Acute Cervical Spine and Spinal Cord Injuries: 2013 Update, in: Clinical Neurosurgery. Congress of Neurological Surgeons, pp. 82–91.

Wang, F., Kameda, M., Yasuhara, T., Tajiri, N., Kikuchi, Y., Liang, H.B., Tayra, J.T., Shinko, A., Wakamori, T., Agari, T., Date, I., 2011. GDNF-pretreatment enhances the survival of neural stem cells following transplantation in a rat model of Parkinson's disease. Neurosci. Res. 71, 92–98. https://doi.org/10.1016/j.neures.2011.05.019

Watanabe, M., Toyama, Y., Nishiyama, A., 2002. Differentiation of proliferated NG2-positive glial progenitor cells in a remyelinating lesion. J. Neurosci. Res. 69, 826–836. https://doi.org/10.1002/jnr.10338

Watson, C., Paxinos, G., Kayalioglu, G., Heise, C., 2009. Atlas of the Rat Spinal Cord, in: The Spinal Cord. Elsevier, pp. 238–306. https://doi.org/10.1016/B978-0-12-374247-6.50019-5

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A.C., Reynolds, B.A., 1996. Multipotent CNS Stem Cells Are Present in the Adult Mammalian Spinal Cord and Ventricular Neuroaxis. J. Neurosci. 16, 7599–7609. https://doi.org/10.1523/JNEUROSCI.16-23-07599.1996

Wells, J.E.A., Hurlbert, R.J., Fehlings, M.G., Yong, V.W., 2003. Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. Brain 126, 1628–1637. https://doi.org/10.1093/brain/awg178

Williams, P.R., Marincu, B.-N., Sorbara, C.D., Mahler, C.F., Schumacher, A.-M., Griesbeck, O., Kerschensteiner, M., Misgeld, T., 2014. A recoverable state of axon injury persists for hours after spinal cord contusion in vivo. Nat. Commun. 5. https://doi.org/10.1038/ncomms6683

Williams, R.R., Henao, M., Pearse, D.D., Bunge, M.B., 2015. Permissive Schwann Cell Graft/Spinal Cord Interfaces for Axon Regeneration. Cell Transplant. 24, 115–131. https://doi.org/10.3727/096368913X674657

Williams, S.K., Franklin, R.J.M., Barnett, S.C., 2004. Response of olfactory ensheathing cells to the degeneration and regeneration of the peripheral olfactory system and the involvement of the neuregulins. J. Comp. Neurol. 470, 50–62. https://doi.org/10.1002/cne.11045

Wilson, J.R., Davis, A.M., Kulkarni, A.V., Kiss, A., Frankowski, R.F., Grossman, R.G., Fehlings, M.G., 2014. Defining age-related differences in outcome after traumatic spinal cord injury: analysis of a combined, multicenter dataset. Spine J. 14, 1192–1198. https://doi.org/10.1016/j.spinee.2013.08.005

Winton, M.J., Dubreuil, C.I., Lasko, D., Leclerc, N., McKerracher, L., 2002. Characterization of New Cell Permeable C3-like Proteins That Inactivate Rho and Stimulate Neurite Outgrowth on Inhibitory Substrates. J. Biol. Chem. 277, 32820–32829. https://doi.org/10.1074/jbc.M201195200

Witiw, C.D., Fehlings, M.G., 2015. Acute spinal cord injury. J. Spinal Disord. Tech. 28, 202–210.

Woltjen, K., Michael, I.P., Mohseni, P., Desai, R., Mileikovsky, M., Hämäläinen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., Kaji, K., Sung, H.-K., Nagy, A., 2009. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458, 766–770. https://doi.org/10.1038/nature07863

Wood, J.G., Byrd, F.I., Gurd, J.W., 1981. Lectin cytochemistry of carbohydrates on cell membranes of rat cerebellum. J. Neurocytol. 10, 149–159. https://doi.org/10.1007/BF01181750

Wright, K.T., Masri, W.E., Osman, A., Chowdhury, J., Johnson, W.E.B., 2011. Concise Review: Bone Marrow for the Treatment of Spinal Cord Injury: Mechanisms and Clinical Applications. STEM CELLS 29, 169–178. https://doi.org/10.1002/stem.570

Xie, Y., Schutte, R.J., Ng, N.N., Ess, K.C., Schwartz, P.H., O'Dowd, D.K., 2018. Reproducible and efficient generation of functionally active neurons from human hiPSCs for preclinical disease modeling. Stem Cell Res. 26, 84–94. https://doi.org/10.1016/j.scr.2017.12.003

Yamashita, T., Fujitani, M., Yamagishi, S., Hata, K., Mimura, F., 2005. Multiple Signals Regulate Axon Regeneration Through the Nogo Receptor Complex. Mol. Neurobiol. 32, 105–112. https://doi.org/10.1385/MN:32:2:105

Yan, J., Xu, L., Welsh, A.M., Hatfield, G., Hazel, T., Johe, K., Koliatsos, V.E., 2007. Extensive Neuronal Differentiation of Human Neural Stem Cell Grafts in Adult Rat Spinal Cord. PLoS Med. 4, e39. https://doi.org/10.1371/journal.pmed.0040039

Yan, S., Li, P., Wang, Y., Yu, W., Qin, A., Liu, M., Xiang, A.P., Zhang, W., Li, W., 2016. Nestin regulates neural stem cell migration via controlling the cell contractility. Int. J. Biochem. Cell Biol. 78, 349–360. https://doi.org/10.1016/j.biocel.2016.07.034

Yang, H.-S., Kwon, H.G., Hong, J.H., Hong, C.P., Jang, S.H., 2011. The rubrospinal tract in the human brain: Diffusion tensor imaging study. Neurosci. Lett. 504, 45–48. https://doi.org/10.1016/j.neulet.2011.08.054

Yick, L.-W., Wu, W., So, K.-F., Yip, H.K., Shum, D.K.-Y., 2000. Chondroitinase ABC promotes axonal regeneration of Clarke's neurons after spinal cord injury 11, 1063–1067.

Yin D., Tavakoli T., Gao WQ., Ma W., 2012. Comparison of Neural Differentiation Potential of Human Pluripotent Stem Cell Lines Using a Quantitative Neural Differentiation Protocol. In: Turksen K. (eds) Human Embryonic Stem Cells Handbook. Methods in Molecular Biology (Methods and Protocols), vol 873. Humana Press, Totowa, NJ

Yrjänheikki, J., Tikka, T., Keinanen, R., Goldsteins, G., Chan, P.H., Koistinaho, J., 1999. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc. Natl. Acad. Sci. 96, 13496–13500. https://doi.org/10.1073/pnas.96.23.13496

Yune, T.Y., Lee, J.Y., Jung, G.Y., Kim, S.J., Jiang, M.H., Kim, Y.C., Oh, Y.J., Markelonis, G.J., Oh, T.H., 2007. Minocycline Alleviates Death of Oligodendrocytes by Inhibiting Pro-Nerve Growth Factor Production in Microglia after Spinal Cord Injury. J. Neurosci. 27, 7751–7761. https://doi.org/10.1523/JNEUROSCI.1661-07.2007

Zelentsova, K., Talmi, Z., Abboud-Jarrous, G., Sapir, T., Capucha, T., Nassar, M., Burstyn-Cohen, T., 2017. Protein S Regulates Neural Stem Cell Quiescence and Neurogenesis: Protein S in NSC Quiescence and Neurogenesis. STEM CELLS 35, 679–693. https://doi.org/10.1002/stem.2522

Zeng, W.-B., Jiang, H.-F., Gang, Y.-D., Song, Y.-G., Shen, Z.-Z., Yang, H., Dong, X., Tian, Y.-L., Ni, R.-J., Liu, Y., Tang, N., Li, X., Jiang, X., Gao, D., Androulakis, M., He, X.-B., Xia, H.-M., Ming, Y.-Z., Lu, Y., Zhou, J.-N., Zhang, C., Xia, X.-S., Shu, Y., Zeng, S.-Q., Xu, F., Zhao, F., Luo, M.-H., 2017. Anterograde monosynaptic transneuronal tracers derived from herpes simplex virus 1 strain H129. Mol. Neurodegener. 12. https://doi.org/10.1186/s13024-017-0179-7

Zhang, J., Jiao, J., 2015. Molecular Biomarkers for Embryonic and Adult Neural Stem Cell and Neurogenesis. BioMed Res. Int. 2015, 1–14. https://doi.org/10.1155/2015/727542

Zhang, J., Liu, X., Zhang, Y., Luan, Z., Yang, Y., Wang, Z., Zhang, C., 2018. Human Neural Stem Cells with GDNF Site-Specific Integration at AAVS1 by Using AAV Vectors Retained Their Stemness. Neurochem. Res. 43, 930–937. https://doi.org/10.1007/s11064-018-2498-7

Zhao, S., Jiang, E., Chen, S., Gu, Y., Shangguan, A.J., Lv, T., Luo, L., Yu, Z., 2016. PiggyBac transposon vectors: the tools of the human gene encoding. Transl. Lung Cancer Res. 5, 6.

Zhou, Z.-D., Kumari, U., Xiao, Z.-C., Tan, E.-K., 2010. Notch as a molecular switch in neural stem cells. IUBMB Life 62, 618–623. https://doi.org/10.1002/iub.362

Ziegler, A.N., Levison, S.W., Wood, T.L., 2015. Insulin and IGF receptor signalling in neural-stem-cell homeostasis. Nat. Rev. Endocrinol. 11, 161–170. https://doi.org/10.1038/nrendo.2014.208

Zimmer, M.B., Nantwi, K., Goshgarian, H.G., 2007. Effect of spinal cord injury on the respiratory system: basic research and current clinical treatment options. Taylor & Francis.

Zörner, B., Bachmann, L.C., Filli, L., Kapitza, S., Gullo, M., Bolliger, M., Starkey, M.L., Röthlisberger, M., Gonzenbach, R.R., Schwab, M.E., 2014. Chasing central nervous system plasticity: the brainstem's contribution to locomotor recovery in rats with spinal cord injury. Brain 137, 1716–1732. https://doi.org/10.1093/brain/awu078

Zörner, B., Schwab, M.E., 2010. Anti-Nogo on the go: from animal models to a clinical trial: Zörner & Schwab. Ann. N. Y. Acad. Sci. 1198, E22–E34. https://doi.org/10.1111/j.1749-6632.2010.05566.x

Zweckberger, K., Ahuja, C.S., Liu, Y., Wang, J., Fehlings, M.G., 2016. Self-assembling peptides optimize the post-traumatic milieu and synergistically enhance the effects of neural stem cell therapy after cervical spinal cord injury. Acta Biomater. 42, 77–89. https://doi.org/10.1016/j.actbio.2016.06.016

Appendices

Supplementary Table 1 Hormone Mix

Reagent	Company	Catalogue No.	Final Concentration
Apotransferrin	Sigma Aldrich	T1147	1 mg/mL
Putrescine	Sigma Aldrich	P5780	20 mM
Progesterone	Sigma Aldrich	P8783	4 µM
Sodium selenite	Sigma Aldrich	S5261	6 µM

Supplementary Table 2 Serum Free Media

Reagent	Company	Catalogue No.	Final Concentration
DMEM/F12 with GlutaMAX	Gibco	10565018	N/A
Hormone Mix	See Above	See Above	See Above
B27 Supplement without Vitamin A	Gibco	12587010	100 X
EGF	Wisent	511-110-EU	20 ng/mL
FGF2	Wisent	511-126-QU	20 ng/mL
Heparin	BioShop	HPA333.100	2 µg/mL
Penicillin-Streptomycin	Gibco	15140122	100 X

Supplementary Table 3 Neural Induction Media

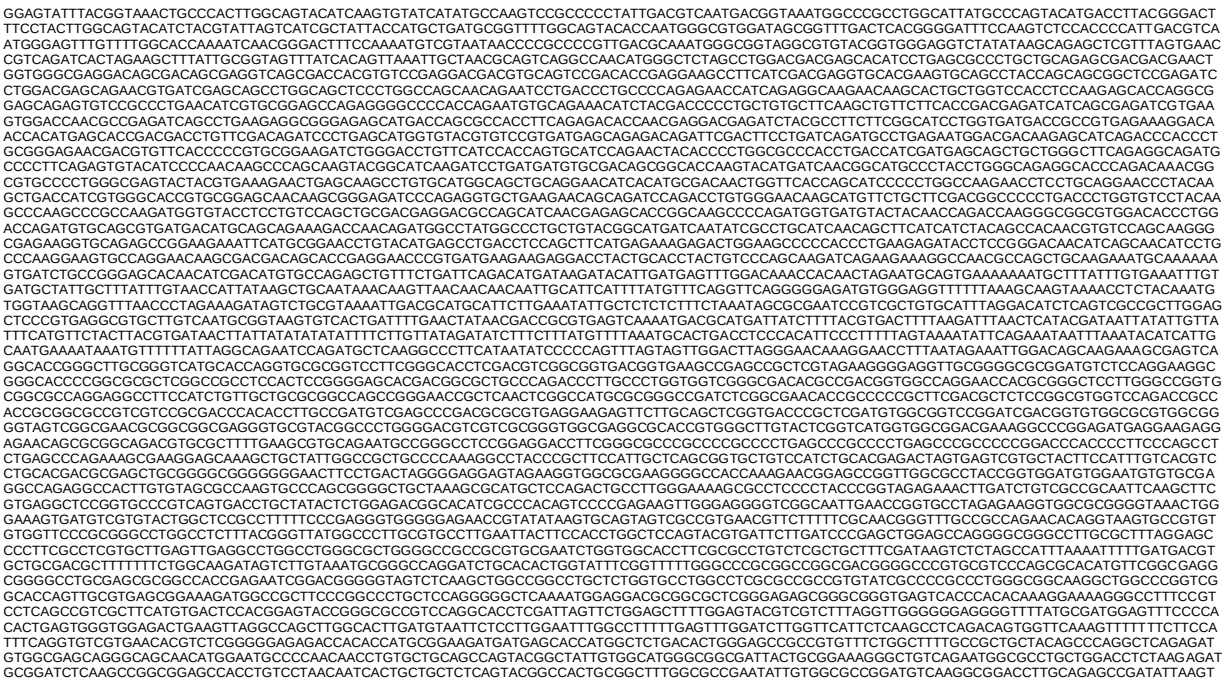
Reagent	Company	Catalogue No.	Final Concentration
Neurobasal Media	Gibco	21103049	N/A
B27 Supplement with Vitamin A	Gibco	17504044	100 X
N2 Supplement	Gibco	17502048	100 X
hBDNF	PeproTech	450-02-10ug	10 ng/mL
Dibutyryl cAMP	Sigma Aldrich	A9501-1G	1 nM
Penicillin-Streptomycin	Gibco	15140122	100 X

Supplementary Table 4 Neural Maintenance Media

Reagent	Company	Catalogue No.	Final Concentration
Neurobasal Media	Gibco	21103049	N/A
B27 Supplement with Vitamin A	Gibco	17504044	100 X
N2 Supplement	Gibco	17502048	100 X
Penicillin-Streptomycin	Gibco	15140122	100 X

Supplementary Table 5 List of Antibodies

Antigen	Company	Catalogue No.	Dilution
TUBB3	Sigma	T2200-200UL	1:200
GFAP	Biolegend	644701	1:200
MAP2	Millipore	AB5622	1:200
NES	Biolegend	656801	1:200
STEM121	Takara	Y40410	1:200
TTC	Antibodies Online	ABIN964673	1:100
WGA	Abcam	ab178444	1:100
DAPI	Invitrogen	D3571	1:1000 to 1:5000
Alexa Fluor 488	Thermo Fisher	A11008	1:1000
Alexa Fluor 568	Thermo Fisher	A11011	1:1000
Alexa Fluor 647	Thermo Fisher	A32728 & A32733	1:1000
Q650	Thermo Fisher	Q11032MP	1:100



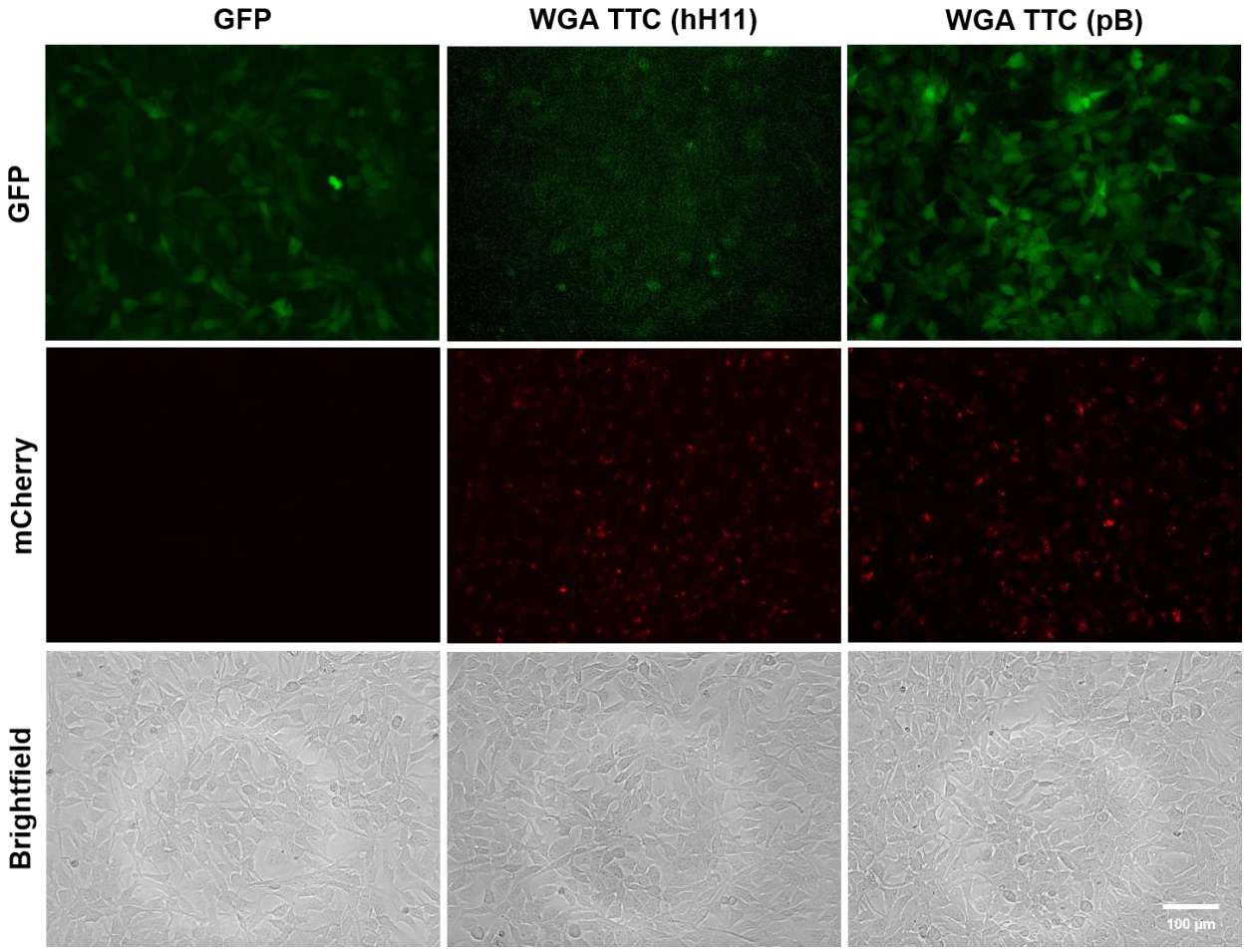
Supplementary Figure 1 CMV::pB//EF1a::WGA-mCherry-IRES Sequence Complete DNA sequence of starting plasmid in a piggyBac backbone.

GGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTCCGCCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTACGGGACT
TTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGCTGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCA
ATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAAC
CGTCAGATCACTAGAAGCTTTATTGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCCAACATGGGCTCTAGCCTGGACGACGAGCACATCCTGAGCGCCCCTGCTGCAGAGCGACGACGAACT
GGTGGGCGAGGACAGCGACAGCGAGGTCAGCGACCACGTGTCCGAGGACGACGTGCAGTCCGACACCGAGGAAGCCTTCATCGACGAGGTGCACGAAGTGCAGCCTACCAGCAGCGGCTCCGAGATC
CTGGACGAGCAGAACGTGATCGAGCAGCCTGGCAGCTCCCTGGCCAGCAACAGAATCCTGACCCTGCCCCAGAGAACCATCAGAGGCAAGAACAAGCACTGCTGGTCCACCTCCAAGAGCACCAGGCG
GAGCAGAGTGTCCGCCCTGAACATCGTGCGGAGCCAGAGGGGCCCCACCAGAATGTCACGAAACATCTACGACCCCCTGCTGTGCTTCAAGCTGTTCTTCACCGACGAGATCATCAGCGAGATCGTGAA
GTGGACCAACGCCGAGATCAGCCTGAAGAGGCGGGAGAGCATGACCAGCGCCACCTTCAGAGACACCAACGAGGACGAGATCTACGCCTTCTTCGGCATCCTGGTGATGACCGCCGTGAGAAAGGACA
ACCACATGAGCACCGACGACCTGTTCGACAGATCCCTGAGCATGGTGTACGTGTCCGTGATGAGCAGAGACAGATTCGACTTCCTGATCAGATGCCTGAGAATGGACGACAAGAGCATCAGACCCACCCT
GCGGGAGAACGACGTGTTCACCCCCGTGCGGAAGATCTGGGACCTGTTCATCCACCAGTGCATCCAGAACTACACCCCTGGCGCCCACCTGACCATCGATGAGCAGCTGCTGGGCTTCAGAGGCAGATG
CCCCTTCAGAGTGTACATCCCCAACAAGCCCAGCAAGTACGGCATCAAGATCCTGATGATGTGCGACAGCGGCACCAAGTACATGATCAACGGCATGCCCTACCTGGGCAGAGGCACCCAGACAAACGG
CGTGCCCCTGGGCGAGTACTACGTGAAAGAACTGAGCAAGCCTGTGCATGGCAGCTGCAGGAACATCACATGCGACAACTGGTTCACCAGCATCCCCCTGGCCAAGAACCTCCTGCAGGAACCCTACAA
GCTGACCATCGTGGGCACCGTGCGGAGCAACAAGCGGGAGATCCCAGAGGGTGCTGAAGAACAGCAGATCCAGACCTGTGGGAACAAGCATGTTCTGCTTCGACGGCCCCCTGACCCTGGTGTCCTACAA
GCCCAAGCCCGCCAAGATGGTGTACCTCCTGTCCAGCTGCGACGAGGACGCCAGCATCAACGAGAGCACCGGCAAGCCCCAGATGGTGATGTACTACAACCAGACCAAGGGCGGCGTGGACACCCTGG
ACCAGATGTGCAGCGTGATGACATGCAGCAGAAAGACCAACAGATGGCCTATGGCCCTGCTGTACGGCATGATCAATATCGCCTGCATCAACAGCTTCATCATCTACAGCCACAACGTGTCCAGCAAGGG
CGAGAAGGTGCAGAGCCGGAAGAAATTCATGCGGAACCTGTACATGAGCCTGACCTCCAGCTTCATGAGAAAGAGACTGGAAGCCCCCACCCTGAAGAGATACCTCCGGGACAACATCAGCAACATCCTG
CCCAAGGAAGTGCCAGGAACAAGCGACGACAGCACCGAGGAACCCGTGATGAAGAAGAGGACCTACTGCACCTACTGTCCCAGCAAGATCAGAAGAAAGGCCAACGCCAGCTGCAAGAAATGCAAAAAA
GTGATCTGCCGGGAGCACAACATCGACATGTGCCAGAGCTGTTTCTGATTCAGACATGAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGT
GATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAGATGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATG
TGGTAAGCAGGTTTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCTTGAAATATTGCTCTCTCTTTCTAAATAGCGCGAATCCGTCGCTGTGCATTTAGGACATCTCAGTCGCCGCTTGGAG
CTCCCGTGAGGCGTGCTTGTCAATGCGGTAAGTGTCACTGATTTTGAACTATAACGACCGCGTGAGTCAAAATGACGCATGATTATCTTTTACGTGACTTTAAGATTTAACTCATACGATAATTATATTGTTA
TTTCATGTTCTACTTACGTGATAACTTATTATATATATTTTCTTGTTATAGATATCTTTCTTTATGTTTTAAATGCACTGACCTCCCACATTCCCTTTTTAGTAAAATATTCAGAAATAATTTAAATACATCATTG
CAATGAAAATAAATGTTTTTTATTAGGCAGAATCCAGATGCTCAAGGCCCTTCATAATATCCCCCAGTTTAGTAGTTGGACCTAGGGAACAAAGGAACCTTTAATAGAAATTGGACAGCAAGAAAGCGAGTCA
GGCACCGGGCTTGCGGGTCATGCACCAGGTGCGCGGTCCTTCGGGCACCTCGACGTCGGCGGTGACGGTGAAGCCGAGCCGCTCGTAGAAGGGGAGGTTGCGGGGCGCGGATGTCTCCAGGAAGGC
GGGCACCCCGGCGCGCTCGGCCGCCTCCACTCCGGGGAGCACGACGGCGCTGCCCAGACCCTTGCCCTGGTGGTCGGGCGACACGCCGACGGTGGCCAGGAACCACGCGGGCTCCTTGGGCCGGTG
CGGCGCCAGGAGGCCTTCCATCTGTTGCTGCGCGGCCAGCCGGGAACCGCTCAACTCGGCCATGCGCGGGCCGATCTCGGCGAACACCGCCCCCGCTTCGACGCTCTCCGGCGTGGTCCAGACCGCC
ACCGCGGCGCCGTCGTCCGCGACCCACACCTTGCCGATGTCGAGCCCGACGCGCGTGAGGAAGAGTTCTTGCAGCTCGGTGACCCGCTCGATGTGGCGGTCCGGATCGACGGTGTGGCGCGTGGCGG
GGTAGTCGGCGAACGCGGCGGCGAGGGTGCGTACGGCCCTGGGGACGTCGTCGCGGGTGGCGAGGCGCACCGTGGGCTTGTACTCGGTCATGGTGGCGGACGAAAGGCCCGGAGATGAGGAAGAGG
AGAACAGCGCGGCAGACGTGCGCTTTTGAAGCGTGCAGAATGCCGGGCCTCCGGAGGACCTTCGGGCGCCCGCCCCGCCCTGAGCCCGCCCCCGGACCCACCCCTTCCCAGCCT
CTGAGCCCAGAAAGCGAAGGAGCAAAGCTGCTATTGGCCGCTGCCCCAAAGGCCTACCCGCTTCCATTGCTCAGCGGTGCTGTCCATCTGCACGAGACTAGTGAGTCGTGCTACTTCCATTTGTCACGTC
CTGCACGACGCGAGCTGCGGGGCGGGGGGGAACTTCCTGACTAGGGGAGGAGTAGAAGGTGGCGCGAAGGGGCCACCAAAGAACGGAGCCGGTTGGCGCCTACCGGTGGATGTGGAATGTGTGCGA
GGCCAGAGGCCACTTGTGTAGCGCCAAGTGCCCAGCGGGGCTGCTAAAGCGCATGCTCCAGACTGCCTTGGGAAAAGCGCCTCCCCTACCCGGTAGAGAAACTTGATCTGTCGCCGCAATTCAAGCTTC
GTGAGGCTCCGGTGCCCGTCAGTGACCTGCTATACTCTGGAGACGGCACATCGCCCACAGTCCCCGAGAAGTTGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGG
GAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAGGTAAGTGCCGTGT
GTGGTTCCCGCGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTGCCTTGAATTACTTCCACCTGGCTCCAGTACGTGATTCTTGATCCCGAGCTGGAGCCAGGGGCGGGCCTTGCGCTTTGAGGAC
CCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCGCTGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTTGATGACGT
GCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTGTAAATGCGGGCCAGGATCTGCACACTGGTATTTCGGTTTTTGGGCCCGCGGCCGGCGACGGGGCCCGTGCGTCCCAGCGCACATGTTCGGCGAGG
CGGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGGCCGGTCG
GCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTCCAGGGGGCTCAAAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCCTTTCCGT
CCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGTCGAGTTGTCTTGGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCCA
CACTGAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCTTTTTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCA
TTTCAGGTGTCGTGAACACGTCTCGGGGGAGAGACCACACCATGCGGAAGATGATGAGCACCATGGCTCTGACACTGGGAGCCGCCGTGTTCTGGCTTTTGCCGCTGCTACAGCCCAGGCTCAGAGAT
GTGGCGAGCAGGGCAGCAACATGGAATGCCCCAACAACCTGTGCTGCAGCCAGTACGGCTATTGTGGCATGGGCGGCGATTACTGCGGAAAGGGCTGTCAGAATGGCGCCTGCTGGACCTCTAAGAGAT
GCGGATCTCAAGCCGGCGGAGCCACCTGTCCTAACAATCACTGCTGCTCTCAGTACGGCCACTCGGCTTTGGCGCCGAATATTGTGGCGCCGGATGTCAAGGCGGACCTTGCAGAGCCGATATTAAGT
GCGGCTCTCAGAGCGGCGGCAAGCTGTGCCCAAACAATCTGTGTTGTTCCCAGTGGGGCTTCTGCGGCCTGGGCTCTGAATTTTGTGGCGGAGGTTGTCAGTCCGGCGCCTGCAGTACAGATAAGCCCT
GTGGAAAAGACGCTGGCGGCAGAGTGTGCACCAACAACTACTGCTGTTCTAAGTGGGGCAGCTGCGGCATCGGCCCTGGATATTGCGGAGCTGGTTGTCAGAGCGGAGGCTGTGATGCTGTGTTTGCCG
CCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCGCTGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTTGATGACGT
GCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTGTAAATGCGGGCCAGGATCTGCACACTGGTATTTCGGTTTTTGGGCCCGCGGCCGGCGACGGGGCCCGTGCGTCCCAGCGCACATGTTCGGCGAGG
CGGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGGCCGGTCG
GCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTCCAGGGGGCTCAAAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCCTTTCCGT
CCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGTCGAGTTGTCTTGGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCCA
CACTGAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCTTTTTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCA
TTTCAGGTGTCGTGAACACGTCTCGGGGGAGAGACCACACCATGCGGAAGATGATGAGCACCATGGCTCTGACACTGGGAGCCGCCGTGTTCTGGCTTTTGCCGCTGCTACAGCCCAGGCTCAGAGAT
GTGGCGAGCAGGGCAGCAACATGGAATGCCCCAACAACCTGTGCTGCAGCCAGTACGGCTATTGTGGCATGGGCGGCGATTACTGCGGAAAGGGCTGTCAGAATGGCGCCTGCTGGACCTCTAAGAGAT
GCGGATCTCAAGCCGGCGGAGCCACCTGTCCTAACAATCACTGCTGCTCTCAGTACGGCCACTCGGCTTTGGCGCCGAATATTGTGGCGCCGGATGTCAAGGCGGACCTTGCAGAGCCGATATTAAGT
GCGGCTCTCAGAGCGGCGGCAAGCTGTGCCCAAACAATCTGTGTTGTTCCCAGTGGGGCTTCTGCGGCCTGGGCTCTGAATTTTGTGGCGGAGGTTGTCAGTCCGGCGCCTGCAGTACAGATAAGCCCT
GTGGAAAAGACGCTGGCGGCAGAGTGTGCACCAACAACTACTGCTGTTCTAAGTGGGGCAGCTGCGGCATCGGCCCTGGATATTGCGGAGCTGGTTGTCAGAGCGGAGGCTGTGATGCTGTGTTTGCCG
GCGCTATCACCGCCAATTCTACCCTGCTGGCTGAAGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCG
AGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAG
GCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCT
GCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCG
CCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAG
TTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGtaaTAGGTCTCTAAAATTCCGCCCCCCCCCT
AACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCT
AGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCAC
CTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATT
CAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTACACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACG
TGGTTTTCCTTTGAAAAACACGATGATAATATGGCCACAACCATGACGGCCCTGACAGAAGGTGCGAAGCTGTTCGAGAAGGAGATTCCCTATATCACAGAATTGGAGGGGGATGTAGAGGGTATGAAGTT
TATCATCAAAGGCGAAGGGACAGGGGATGCAACAACTGGAACAATTAAGGCTAAGTACATTTGCACGACCGGCGACGTCCCGGTGCCCTGGTCCACGCTCGTCACCACGCTCACGTACGGAGCCCAGTG
CTTTGCCAAATATGGCCCTGAACTTAAAGACTTCTACAAGTCGTGTATGCCGGAGGGATACGTGCAAGAGAGGACGATCACCTTTGAAGGTGACGGAGTATTCAAAACAAGAGCGGAGGTGACGTTCGAGA
ATGGATCGGTCTATAACCGGGTCAAGCTCAACGGACAGGGCTTTAAGAAAGATGGACACGTCCTTGGGAAGAATTTGGAGTTCAATTTCACCCCGCATTGTCTTTACATCTGGGGTGATCAGGCGAATCAC
GGGTTGAAATCAGCGTTCAAGATCATGCACGAGATTACGGGGAGCAAAGAGGACTTTATCGTGGCAGACCACACTCAGATGAACACTCCAATCGGAGGGGGTCCCGTACACGTACCCGAGTATCATCACCC
TGACCGTCTGGACATCGTTTGGAAAAGACCCTGACGACGATGAAACTGATCATCTCAACATTGTGGAAGTGATCAAGGCGGTGGACTTGGAAACATACCGGATGAAGAACCTGGACTGCTGGGTTGACAAC
GAAGAGGACATCGACGTGATCCTGAAGAAGTCCACCCATCCTGAATCTGGACATCAACAACGACATTAACATCGACATCAGCGGCTTCAACAGCAGCGTGATCACATACCCCGACGCTCAGCTGGTGCCTG
GCATCAATGGAAAGGCCATCCACCTGGTCAACAATGAGTCCAGCGAAGTGATCGTGCACAAGGCCATGGACATCGAGTACAACGATATGTTCAACAACTTCACCGTGTCCTTTTGGCTGCGGGTGCCCAAA
GTGTCTGCCAGCCACCTGGAACAGTACGGCACCAACGAGTACAGCATCATCTCCAGCATGAAGAAGCACAGCCTGAGCATCGGAAGCGGCTGGAGCGTTAGCCTGAAGGGCAACAATCTGATCTGGACC
CTGAAGGACTCTGCCGGCGAAGTGCGGCAGATCACCTTAGGGACCTGCCTGACAAGTTCAACGCCTACCTGGCCAACAAATGGGTGTTCATCACCATCACCAACGACCGGCTGAGCAGCGCCAACCTGT
ACATCAATGGGGTGCTGATGGGCAGCGCCGAGATCACAGGACTGGGAGCTATCAGAGGACAACAACATCACCCTGAAACTGGACCGGTGCAACAACAACAATCAGTACGTGTCCATCGACAAGTTTCG
GATCTTCTGCAAGGCTCTGAACCCCAAAGAGATCGAGAAGCTGTATACCAGCTACCTGAGCATTACCTTTCTGCGGGACTTCTGGGGCAACCCTCTGAGATACGACACCGAGTACTATCTGATCCCCGTGG
CCTCCAGCAGCAAGGACGTGCAGCTGAAGAACATCACCGACTACATGTACCTGACAAACGCCCCTAGCTACACCAACGGAAAGCTGAACATCTACTACCGGCGGCTGTACAACGGCCTGAAGTTCATCAT
CAAGCGGTACACCCCTAACAACGAGATCGACAGCTTCGTGAAGTCCGGCGACTTTATCAAGCTGTACGTCAGCTATAACAACAACGAGCACATCGTGGGCTACCCCAAGGACGGCAACGCCTTTAACAAC
CTGGACAGAATCCTGAGAGTGGGCTACAACGCCCCTGGCATCCCTCTGTACAAAAAGATGGAAGCCGTGAAGCTGCGGGACCTGAAAACCTACAGCGTCCAGCTGAAACTGTACGACGACAAGAACGCCT
CTCTGGGCCTCGTGGGCACACACAATGGCCAGATCGGCAACGACCCCAACCGGGACATTCTGATCGCCAGCAACTGGTACTTCAACCACCTGAAGGATAAGATCCTGGGCTCGCGATTGGTACTTCGTGCC
CACCGATGAAGGCTGGACCAACGATTAATAGTGATTCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTA
TTTGTAACCATTATAAGCTGCAATAAACAAGTTGTCGACCATGGAACGAGGTTAAGGTCTCTAAAGCACAGTACATGGACAGATATCCACGTAGATATTACGAGACGATTGAGACGATAGCCAGTATGACGA
TAGACGATATGGATAGACCATGCTTACCCGGTAAGCCAATCGGGTATACACGGTCGTCATACTGCAGACAGGGTTCTTCTACTTTGCAAGATAGTCTTGAGTAGTAAAATAAATAGATAGAGAAAAGCGTCT
TCCTGTTCTCATCACATCATATCAAGGTTATATACCATCAATATTGCCACAGATGTTACTTAGCCTTTTAATATTTCTCTAATTTAGTGTATATGCAATGATAGTTCTCTGATTTCTGAGATTGAGTTTCTCATGT
GTAATGATTATTTAGAGTTTCTCTTTCATCTGTTCAAATTTTTGTCTAGTTTTATTTTTTACTGATTTGTAAGACTTCTTTTTATAATCTGCATATTACAATTCTCTTTACTGGGGTGTTGCAAATATTTTCTGTCAT
TCTATGGCCTGACTTTTCTTAATGGTTTTTTAATTTTAAAAATAAGTCTTAATATTCATGCAATCTAATTAACAATCTTTTCTTTGTGGTTAGGACTTTGAGTCATAAGAAATTTTTCTCTACACTGAAGTCATGAT
GGCATGCTTCTATATTATTTTCTAAAAGATTTAAAGTTTTGCCTTCTCCATTTAGACTTATAATTCACTGGAATTTTTTTGTGTGTATGGTATGACATATGGGTTCCCTTTTATTTTTTACATATAAATATATTTCC
CTGTTTTTCTAAAAAAGAAAAAGATCATCATTTTCCCATTGTAAAATGCCATATTTTTTTCATAGGTCACTTACATATATCAATGGGTCTGTTTCTGAGCTCTACTCTATTTTATCAGCCTCACTGTCTATCCCCA
CACATCTCATGCTTTGCTCTAAATCTTGATATTTAGTGGAACATTCTTTCCCATTTTGTTCTACAAGAATATTTTTGTTATTGTCTTTGGGCTTTCTATATACATTTTGAAATGAGGTTGACAAGTTAATAATCAAC
CTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTTAACTATGTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTTCTCCTCCT
TGTATAAATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGCTGACGCAACCCCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTT
TCCGGGACTTTCGCTTTCCCCCTCCCTATTGCCACGGCGGAACTCATCGCCGCCTGCCTTGCCCGCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAATTCCGTGGTGTTGTCGGGGAAATCATCG
TCCTTTCCTTGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGGCTCTGCGGCCTCTTC
CGCCTCTTCGCCTTCGCCCTCAGACGAGTCGGATCTCCCTTTGGGCCGCCTCCCCGCATCGCCTGCTATTGTCTTGCCAATCCCTCCCCCTTGCTGTCCTCCCCCCCCAAAAACACCACCCCCAGAATAGAATGACA
CCTACTCAGACAATGCGATGCAATTTCCTCATTTTATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGCACGGGGGAGGGGCAAACAACAGATGGCTGGCAACTAGAAGGCACATTT
GTTACTTTATAGAAGAAATTTGAGTTTTTGTTTTTTAATAAATAAATAACATAAATAAAATTGTTTGTTGAATTTATTATTAGTATGTAAATATAATAAAACTTAATATCTATTCAAATTAATAAATAA
ACCTCGATATACAGACCGATAAAACACATGCGTCAATTTTACGCATGATTATCTTTAACGTACGTCACAATATGATTATCTTTCTAGGGTTAAGAAGACTGTCAGAATTGGTTAATTGGTTGTAACACTGACCC
CTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAATATGAGCCATATTCAACGGGAAACGTCGAGGCCGCGATTA
AATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGCTTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGG
TAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCACTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGG
AAAAACAGCGTTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCACTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTT
CGCCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAACTTTTGCCATTCTCACCGGA
TTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACTGC
CTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAACTCATGACCAAAATCCCTTAACGT
GAGTTACGCGCGCGTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTT
GTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGCCCACCACTTCAAGAACTCTGTAGCACCGCCTA
CATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGT
GCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGA
ACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAA
AACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCTC
AATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATTGGCTCATGTCCAATATGACCGCCATGTTG
GCATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCC
ATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGT

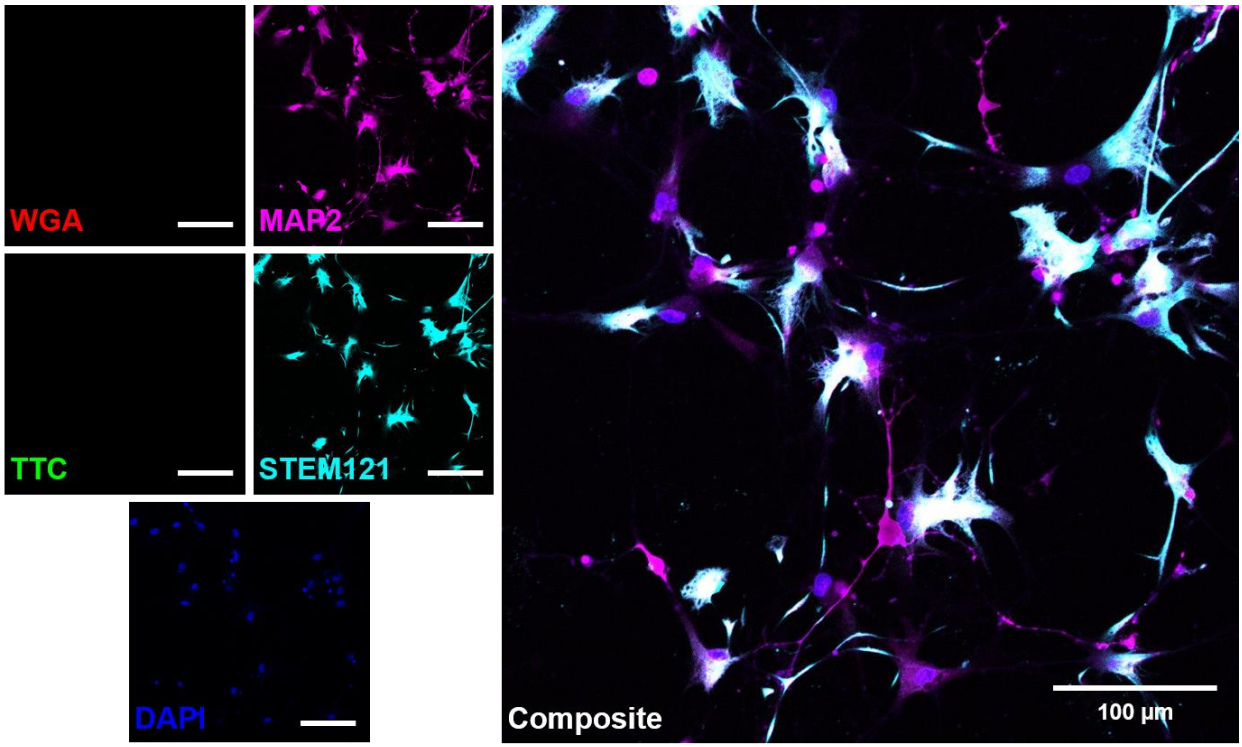
Supplementary Figure 2 WGA TTC (pB) Sequence Complete DNA sequence of self-tracing plasmid in a piggyBac backbone.

CCAGGCCTCATATGATCGATTAATTAACCGGTCCTAGGAAGgtAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTTGGGGTCTTTAAAGCTCAAGGAAAAAGGCCATAGTTGATTTCTCCTAAATCAAGA
TAGAGTCCAATTAACTTTTTTTTTTTTTTTAAGATGGAGTCTCACTCTGTCGCCAGGCTGGACTGCAGTGGCGTGATCTCAGCTCACTGCAACCTCTGCCTCCCAGGTTCAGGCGATTCTTCTGTCTCAGCCTCCCGAGT
AGCTGGGACTACAAGTGTGGGCCACCATGCCCAGCTAGTTTTGTATTTTTAGTAGAGATGGGATTTCACCATCTTGGCTAGGATGGTCTTGATCTCTTGACCTTGTGATTCGCCTGCCTCTGCCTCCCAGAGTGCTGA
GATTACAGGCGTGAGCCACAGTGCCCAATTTCCATGGGTTTTCAAGAAAAACTTAACTTACTTAAACTTCAGATCACTTCCTTAAAGGAACATACTGAGCATAGCTTGGCTGGTTAGAAATTAGGAAGACTAGCTTGGG
CTACATGGTAAGACCCTGCCTCTACAAAAAATAAGAAAAAAGTTAGTGGCATGTGCCTGTAGTTCCATCTACTTGGGAGGCTGAGGTGAGAGGATCGCTTGAGCCCAGGAGGTTGAAGCTGCAGTGAGCCACGATTG
CACCACTGTACTCCAGCCTGGGTGACACAGAGTGAGACCCTGTCTCCAAACAAAATTAGGAAGGGTTTCAGAGAGGAAATAAACACAACAATGTCAGTTACTTCATTCACACACTTAGCATATGCAATTTTAATCAACC
AAAAGAAAATCCCAAATGTACAAGTGAAAAAAATCCAAACTGTTGACACAGGCCTAACTAATATCAGCTACTTTTATGTACAGCTGTATAGTTCAAAAAAGCTATCCTATATGTATATACAAAAGTTGTTTATACACAGGT
CTGTACATAAGGGTCTATACATTTATTTCCTCAGAACCCTTAGGTGCCACCTCTTGGTGAGGACACCAACACTTCATTCACATATCTTACAAAAAAGAAAGACCATTTCCAGGATTGACAACATTGTGCATCCTGTATTC
AGACAATGTACACTCCACTCGACTGGTTAAGATCCTTCCATCATAATGCGGACCACACCAGATGCATGGTTCCACCAAACAGCTTCACACAGAGTGCTCCAAAGTAAAAGCCCATAAACCAACCCGAGATGAAGCAG
GGTCCTGCCCAGTGTGCCACCACAGGTCCGGGCTTAGTTGAGTCTGCCTGCCCTGCTCACTGTCGGTATTCCAATTCATCTACACTGATAACTTTGGCGGGCATGGAGGGCAGGGGTTGCTGTAGCACATCTGAGC
CAAACCATTTGGCAAGGCCCACAGGGGAGCTGCTCCTCTGGCTGGGGCGATGCTCCAGCTGGGAGTGCATGTGGGGCAGGCCTGACCGGCTGGGCACGTTCTGAAGGGTTGTCTGAACGCTGACAGCTGCTGCA
TGGGAACCAGAGCCTGGAGGATGCAGAACTGTGGAGATAAGAAGGGTTATCATTCACAGCATGAGAAGGAAAGTTTATTAGACCCTGCTTCATTCAAACCCTATCCAAAGAAAAGTCTTTACTAGAGGATTTTTTCTAG
GGCTTCTTTAGAAACTCCTTTGCAAAATACCAACAATGCCAGAAAACAGTACCATGGAAAGCTGAGCTATTCAGAAGCTACCAGTATTGAAAACCCACCCTGGATGACATGACCGCTACTTCTCATGGCTAAGTAACCA
CATCACAGAAGCAGCAACATTAACTGGTGAGGGGGTATATACCCTGGGCAGGCCGGATGCTGAACCTCTGTTGGATTCTCAGCCCTCTCTCCTAACAGGCAAAGGAAAAAGCCAGAAAACACTTGGCTTTTCCCACT
GACTTTGCAGGAACAGTAATCCCAAATTGCATAGAGGTCAATTTACTTCATCTTATTATCTGGTTACACTTTGTCCCAAACTTAGAACAAGTTAAAGAATGAAAGTGTTAATTCATCTCCACTGGAAACTAGTCCTCAAAG
CTTCAAGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGT
AATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCC
AGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAA
CTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATT
CTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGT
CCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGA
CAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGAT
GAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGC
GCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGA
AGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCCCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG
CCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGC
GCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACAT
AGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAATTG
AAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG
GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAA
CTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAAC
TTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGAT
GCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTG
GCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACG
AAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAAGTTATCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAAT
CTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAG
CGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGC
CTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCAC
ACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGC
GCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGC
CTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAG
CGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAA
TGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGA
GCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTATGCAGTCAAAGGCTTCTCCTCATTAACTCTAAGACTTTACTGGTTTGGCTGTCATAGCATGATTTGAATTTCCTGTGCTTTTACATATTCC
ATGTTTCTTTCCTGACTTTATTACAAGTAAACAGATCCATTGTTCTGTGCCATTTAACTAGTTAGAACACGTTTAGCTGCATTTAGCAGAAAGTGCTAACTTAAGCGGCTGAAATGGAAGGGAACTTGGTCTTACCTTTTT
GGGAAGCTGTTTTCCAGAAGCCACCCAGTTTGTTGTCTCGTGGCCTAAAATTGGGTCACATGTCTATTTTTGAATCAATTGTTTGGTAAAGGGAATAGAATTATTGAAGAACCAGAATTTAACCCTGTAAGTTATCGTCAT
TCTTGGTTTGTGTTTATGCTCATAGTTCACAAAGGCCAATGGACCTCCCACTTCCCTTCCCTGTTAATAGAGTCTGGCTTAAAAGACACTCAGTAGCTATCTCCTAGGGGAAGGCCTAGCTGCACTCTCTGTTCACTTC
ATTCCCACTTCCTTTTACCACTGACCTCCCCACAGACTTATTTAACTGCTTCTTTGGGAGGGACTATTCTTTCCTCTCCTACCTGACCTCCCCTGCTTAACAGATTTTCCATGATATGCTTTGTGGTCCTCAGCTGTTTTA
CTTTCAGGATGCCTTCTATATCCTCAGCTTCTCTTAACTTTATTTTGCACATCCTTCCAGCACTGATTGCTTGTGAACTCATCTCGTGCCTGCAAGACATCAAAAGTTTCTCCAAAGACATCCCTTCCTCTCTGCCCTCA
TCCTATATCAAGGTCTTAGCTCCTTGAAGACTGCATTAATGTGTCTTTTGCCTTCATCCCCCCCCCACCCTCCCGCTTCCTCTAGTCCTTGTGTTTACCAAAATACTTTTGCTAAAACCTGTATGTCTAGCTTCTGCTGAT
ACTCTTAGCAATACTCCCTTATTTCCTTCTGGCGCCATTTGCCAATCACCAGCTAATGGCTTTGCTCTTTGCATGGTACCTGTTTCTGTCCTACTAGCTTCTAGTCTGAAAAGGAAGCCTTTACTAGCAGCTTCAACCCA
GTCCTCCTTACCTTGCACCTATTAACGTTGGTTCAACATGAAGGGGAAGTAAGATAAGTGGAAGAGGATACACAGGAAAGGTGGAACTGGATTCAGGTATCTGTGTTCTGCCATTGACACTTGGGACTGCGTTCCAGC
TTCCACCTGTGGAAACCAGAAAGCATTTGCCAAAAATTCTAAACAGAGGGTCTATTTCTGAAGCTGAGGAATCACATGGAGTGAATAGCATGGGGGAAGGGGATTACTAATGAGGCTGAAAGAAAAGTACCAGGAAGA
GACTGACTTCATGCATAATCCATAAAGCCAGGTTTTCTTGAAAGGTGACCTGCTTTCTTGTATTCCTGCAAGTACACGGCAGGGTGGGCAGTTCCAGGCTTATAGTCATTATTCCCTAACAAACGGTCAAGACTTAGGC
TGCCCAAGACTCTACTACCCTTGTAGTCAAAAATCCAACCACCTTGACCTTACTTCATTATCTGACCCTTTAGCTGGAGTCCTAAAATGGTCCTGAATGGACAAATATCTAGAACTCTGGTAAATTAACTTGAGTAGC
ATGGGAAATAAGTATCACCTCATAGCAGGACTGAAAAACCTCCAGTTAGGAAAGGGGACTCAGAACTGTACTGTATTCTGTAGAATCTTTATCCGTAAGTGCTATGTATATACTAATAGTTAAGGTGAATAATAAAACAA
TTGTGAGCTAAGGAATGTGATACAGAACACAAGGAGTCAATTAATGCATGAGTCTTAAGGGAATTCTTAGACTTCAGTGGGTCTAAGCTCAGAACTCTGAGCTTAGAGAAACAGGATGCCCCAGTAAACATGATTTGT
TTGAGAGAAACTACCATATGTAATAGATGATTCCTCAATAAACTGTTTTCACTAGGTCAGTTTAATCTTGGAAATTACACTATAGATATCCTGGTCCGCGGTCGACGGATCGATCGCGGCATATCATGCGACTGATCGAT
CGCAATTATATCAGACCCGCGATCGCGACGTCGATCGATGCATGCAAATGTAGCTAACTATCATCGCACGGGCAGTGTGCATGCATCAACATGATCATATCGCGGTAGCTACGATGCTCGATCGATGCTAGCAGCTC
CCGTGAGGCGTGCTTGTCAATGCGGTAAGTGTCACTGATTTTGAACTATAACGACCGCGTGAGTCAAAATGACGCATGATTATCTTTACGTGACTTTTAAGATTTAACTCATACGATAATTATATTGTTATTTCATGTTC
TACTTACGTGATAACTTATTATATATATATTTTCTTGTTATAGATATCCTTTCTTTATGTTTTAAATGCACTGACCTCCCACATTCCCTTTTTAGTAAAATATTCAGAAATAATTTAAATACATCATTGCAATGAAAATAAATGTT
TTTTATTAGGCAGAATCCAGATGCTCAAGGCCCTTCATAATATCCCCCAGTTTAGTAGTTGGACTTAGGGAACAAAGGAACCTTTAATAGAAATTGGACAGCAAGAAAGCGAGTTCAGGACCGGGCTTCGGGGTCATG
CACCAGGTGCGCGGTCCTTCGGGCACCTCGACGTCGGCGGTGACGGTGAAGCCGAGCCGCTCGTAGAAGGGGAGGTTGCGGGGCGCGGATGTCTCCAGGAAGGCGGGCACCCCGGCGCGCTCGGCCGCCTCC
ACTCCGGGGAGCACGACGGCGCTGCCCAGACCCTGCCCTGGTGGTCGGGCGACACGCCGACGGTGGCCAGGAACCACGCGGGCTCCTTGGGCCGGTCGCGCCAGGAGGCCTTCCATCTGTTGCTGCCGCGG
CCAGCCGGGAACCGCTCAACTCGGCCATGCGCGGGCCGATCTCGGCGAACACCGCCCCCGCTTCGACGCTCTCCGGCGTGGTCCAGACCGCCACCGCGGCCGTCGTCCGCGACCCACACCTTGCCGATGTC
GAGCCCGACGCGCGTGAGGAAGAGTTCTTGCAGCTCGGTGACCCGCTCGATGTGGCGGTCCGGATCGACGGTGTGGCGCGTGGCGGGGTAGTCGGCGAACGCGGCGGCGAGGGTGCGTACGGCCCTGGGGAC
GTCGTCGCGGGTGGCGAGGCGCACCGTGGGCTTGTACTCGGTCATGGTGGCGGACGAAAGGCCCGGAGATGAGGAAGAGGAGAACAGCGCGGCAGACGTGCGCTTTTGAAGCGTGCAGAATGCCGGGCCTCCG
GAGGACCTTCGGGCGCCCGCCCCGCCCTCGAGCCCGCCCCTGAGCCGAAGGGCCGACCGCGTCCCAGCCTCTGAGCCCAGAAAGCGAAGGAGCAAAGCTGCTATTGGCCGCTGCCCAAAGGCCTACCCG
CTTCCATTGCTCAGCGGTGCTGTCCATCTCGCACGAGACTAGTGAGTCGTGCTACTTCCATTTGTCACGTCCTGCACGACGCGAGCTGCGGGGCGGGGGGGAACTTCCTGACTAGGGGAGGAGTAGAAGGTGGCGC
GAAGGGGCCACCAAAGAACGGAGCCGGTTGGCGCCTACCGGTGGATGTGGAATGTGTGCGAGGCCAGAGGCCACTTGTGTAGCGCCAAGTGCCCAGCGGGGCTGCTAAAGCGCATGCTCCAGACTGCCTTGGGA
AAAGCGCCTCCCCTACCCGGTAGAGAAACTTGATCTGTCGCGCAATTCAAGCTTCGTGAGGCTCCGGTCCGTCAGTGACCTGCTATACTCTGGAGACGGCATCGCCCACAGTCCCCGAGAAGTTGGGGAGGG
GTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTT
TTCGCAACGGGTTTGCCGCCAGAACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTGCCTTGAATTACTTCCACCTGGCTCCAGTACGTGATTCTTGATCCCGAG
CTGGAGCCAGGGGCGGGCCTTGCGCTTTAGGAGCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCGCTGCTTTCGATAAG
TCTCTAGCCATTTAAAATTTTTGATGACGTGCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTGTAAATGCGGGCCAGGATCTGCACACTGGTATTTCGGTTTTGGGCCCGCGGCCGGCGACGGGGCCCGTGCGTC
CCAGCGCACATGTTCGGCGAGGCGGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTCACTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCGCCCGCCCTGGGCGGC
AAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTCCAGGGGGCTCAAAATGGAGGACGCGGCGCTCGGGAGAAGGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGG
GCCTTTCCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGTCCAGGCACCTCGATTAGTTCTGGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGGAGGGGTTTTATGCGATGGAGTTTC
CCCACACTGAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCCTTTTTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCATTT
CAGGTGTCGTGAACACGTCTCGGGGGAGAGACCACACCATGCGGAAGATGATGAGCACCATGGCTCTGACACTGGGAGCCGCCGTGTTTCTGGCTTTTGCCGCTGCTACAGCCCAGGCTCAGAGATGTGGCGAGC
AGGGCAGCAACATGGAATGCCCCAACAACCTGTGCTGCAGCCAGTACGGCTATTGTGGCATGGGCGGCGATTACTGCGGAAAGGGCTGTCAGAATGGCGCCTGCTGGACCTCTAAGAGATGCGGATCTCAAGCCG
GCGGAGCCACCTGTCCTAACAATCACTGCTGCTCTCAGTACGGCCACTGCGGCTTTGGCGCCGAATATTGTGGCGCCGGATGTCAAGGCGGACCTTGCAGAGCCGGCATTAAGTGCGGCTCTCAGAGCGGCGGCA
AGCTGTGCCCAAACAATCTGTGTTGTTCCCAGTGGGGCTTCTGCGGCCTGGGCTCTGAATTTTGTGGCGGAGGTTGTCAGTCCGGCGCCTGCAGTACAGATAAGCCCTGTGGAAAAGACGCTGGCGGCAGAGTGTG
CACCAACAACTACTGCTGTTCTAAGTGGGGCAGCTGCGGCATCGGCCCTGGATATTGCGGAGCTGGTTGTCAGAGCCGAGGCTGTGATGCTGTTTGCCGGCGCTCATCACCGCCAATTCTACCCTGCTGGCTGAA
GTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCAC
CCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTC
CTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTC
CGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCT
GAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGC
CACTCCACCGGCGGCATGGACGAGCTGTACAAGtaaTAGGTCTCTAAAATTCCGCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCC
GTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTT
GAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGA
GTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGA
GGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATATGGCCACAACCATGACGGCCCTGACAGAAGGTGCGAAGCTGTTCGAGAAGGAGATTCCCTATATCACA
GAATTGGAGGGGGATGTAGAGGGTATGAAGTTTATCATCAAAGGCGAAGGGACAGGGGATGCAACAACTGGAACAATTAAGGCTAAGTACATTTGCACGACCGGCGACGTCCCGGTGCCCTGGTCCACGCTCGTCA
CCACGCTCACGTACGGAGCCCAGTGCTTTGCCAAATATGGCCCTGAACTTAAAGACTTCTACAAGTCGTGTATGCCGGAGGGATACGTGCAAGAGAGGACGATCCACCTTTGAAGGTGACGGAGTATTCAAAACAAGA
GCGGAGGTGACGTTCGAGAATGGATCGGTCTATAACCGGGTCAAGCTCAACGGACAGGGCTTAAGAAAGATGGACACGTCCTTGGGAAGAATTTGGAGTTCAATTTCACCCCGCATTGTCTTTACATCTGGGGTGA
TCAGGCGAATCACGGGTTGAAATCAGCGTTCAAGATCATGCACGAGATTACGGGGAGCAAAGAGGACTTTATCGTGGCAGACCACACTCAGATGAACACTCCAATCGGAGGGGGTCCCGTACACGTACCCGAGTAT
CATCACCTGACCGTCTGGACATCGTTTGGAAAAGACCCTGACGACGATGAAACTGATCATCTCAACATTGTGGAAGTGATCAAGGCGGTGGACTTGGAAACATACCGGATGAAGAACCTGGACTGCTGGGTTGACAA
CGAAGAGGACATCGACGTGATCCTGAAGAAGTCCACCATCCTGAATCTGGACATCAACAACGACATTAACATCGACATCAGCGGCTTCAACAGCAACCATACCCCGACGCTCAGCTGGTGCCTGGCATCA
ATGGAAAGGCCATCCACCTGGTCAACAATGAGTCCAGCGAAGTGATCGTGCACAAGGCCATGGACATCGAGTACAACGATATGTTCAACAACTTCACCGTGTCCTTTTGGCTGCGGGTGCCCAAAGTGTCTGCCAGC
CACCTGGAACAGTACGGCACCAACGAGTACAGCATCATCTCCAGCATGAAGAAGCACAGCCTGAGCATCGGAAGCGGCTGGAGCGTTAGCCTGAAGGGCAACAATCTGATCTGGACCCTGAAGGACTCTGCCGGC
GAAGTGCGGCAGATCACCTTTAGGGACCTGCCTGACAAGTTCAACGCCTACCTGGCCAACAAATGGGTGTTCATCACCATCACCAACGACCGGCTGAGCAGCGCCAACCTGTACATCAATGGGGTGCTGATGGGCA
GCGCCGAGATCACAGGACTGGGAGCTATCAGAGAGGCAACAACATCACCCTGAAACTGGACCGGTGCAACAACACAATCAGTACGTGTCCATCGACAAGTTTCGGATCTTCTGCAAGGCTCTGAACCCCAAAGAG
ATCGAGAAGCTGTATACCAGCTACCTGAGCATTACCTTTCTGCGGGACTTCTGGGGCAACCCTCTGAGATACGACACCGAGTACTATCTGATCCCCGTGGCCTCCAGCAGCAAGGACGTGCAGCTGAAGAACATCAC
CGACTACATGTACCTGACAAACGCCCCTAGCTACACCAACGGAAAGCTGAACATCTACTACCGGCGGCTGTACAACGGCCTGAAGTTCATCATCAAGCGGATCGACAGCTTCGTGAAGT
CCGGCGACTTTATCAAGCTGTACGTCAGCTATAACAACAACGAGCACATCGTGGGCTACCCCAAGGACGGCAACGCCTTTAACAACCTGGACAGAATCCTGAGAGTGGGCTACAACGCCCCTGGCATCCCTCTGTAC
AAAAAGATGGAAGCCGTGAAGCTGCGGGACCTGAAAACCTACAGCGTCCAGCTGAAACTGTACGACGACAAGAACGCCTCTCTGGGCCTCGTGGGCACACACAATGGCCAGATCGGCAACGACCCCAACCGGGAC
ATTCTGATCGCCAGCAACTGGTACTTCAACCACCTGAAGGATAAGATCCTGGGCTGCGATTGGTACTTCGTCGCCACCGATGAAGGCTGGACCAACGATTAATAGTGATTCAGACATGATAAGATACATTGATGAGTT
TGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTGTCGACCATGGAACGAGGTTAAGGTCTCTAAAGCACA
GTACATGGACAGATATCCACGTAGATATTACGAGACGATTGAGACGATAGCCAGTATGACGATAGACGATATGGATAGACCATGCTTACCCGGTAAGCCAATCGGGTATACACGGTCGTCATACTGCAGACAGGGTT
CTTCTACTTTGCAAGATAGTCTTGAGTAGTAAAATAAATAGATAGAGAAAAGCGTCTTCCTGTTCTCATCACATCATATCAAGGTTATATACCATCAATATTGCCACAGATGTTACTTAGCCTTTTAATATTTCTCTAATTTA
GTGTATATGCAATGATAGGTTCTGTGATTTCTGAGATTGAGTTTCTCATGTGTAATGATTATTTAGAGTTTCTCTTTCATCTGTTCAAATTTTTGTCAGTTTTATTTTTCTACTGATTTGTAAAGACTTCTTTTTATAATCTGCAT
ATTACAATTCTCTTTACTGGGGTGTTGCAAATATTTTCTGTCATTCTATGGCCTGACTTTTCTTAATGGTTTTTTATTTTAAAAATAAGTCTTAATATTCATGCAATCTAATTAACAATCTTTTCTTTGTGGTTAGGACTTTG
AGTCATAAGAAATTTTTTCTCTACACTGAAGTCATGATGGCATGCTTCTATATTATTTTCTAAAAGATTTAAAGTTTTGCCTTCTCCATTTAGACTTATAATTCACTGGAATTTTTTGTTGTGATGGTATGACATATGGGTTC
CCTTTTATTTTTTACATATAAATATATTTCCCTGTTTTTCTAAAAAAGAAAAAGATCATCATTTTCCCATTGTAAAATGCCATATTTTTTTCATAGGTCACTTACATATATCAATGGGTCTGTTTCTGGTACACATTCGGCATC
GACGATGTAATCGATCGATCGATGCTGGCTATCTATAGCACAGACAGACAATATGGGCGCTTACGCGATATATGCGGATTCGACGACTTCGATCGACGCATATGTATCACATACATGCGCGCATCGACTCTGATGCAT
CGTCGCCAACTCGTACACGCG

Supplementary Figure 3 WGA TTC (hH11) Sequence Complete DNA sequence of self-tracing plasmid with tracers between homologous H11 regions.



Supplementary Figure 4 GFP and mCherry expression in control and self-tracing NSCs
Fluorescent protein expression in self-tracing NSCs was examined over multiple passaged and briefly compared to control GFP NSCs. Self-tracing NSCs show clear WGA-mCherry expression in a punctate patter. GFP-TTC is also seen distributed throughout the cytosol. Self-tracing (pB) cells show greater GFP expression.



Supplementary Figure 5 Wild-type NSCs serve as negative control for GFP and mCherry signal seen in co-culture Wild-type NSCs do not display any GFP or mCherry signal, even when stained with WGA and TTC and appropriate secondary antibodies. Primary rat neurons do not show any tracer uptake either.

Copyright Acknowledgements

None to declare.