Developing High Purity Embryonic Stem Cell-Derived V2a Interneurons for In Vitro Investigation and Transplantation Following Spinal Cord Injury

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Developing High Purity Embryonic Stem Cell-Derived V2a Interneurons for \textit{In Vitro} Investigation and Transplantation Following Spinal Cord Injury

by

Nisha Iyer

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
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of Doctor of Philosophy

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Nisha Iyer

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December 2016
Like the entomologist in search of colorful butterflies, my attention has chased in the gardens of the grey matter cells with delicate and elegant shapes, the mysterious butterflies of the soul, whose beating of wings may one day reveal to us the secrets of the mind.—Ramón Y Cajal

Dedicated to my grandfather, Dr. T.N. Ananthakrishnan, the great entomologist, who showed me what was achievable.
ABSTRACT OF THE DISSERTATION

Developing High Purity Embryonic Stem Cell-Derived V2a Interneurons for In Vitro Investigation and Transplantation Following Spinal Cord Injury

By Nisha Iyer

Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, 2016

Professors Shelly Sakiyama-Elbert & James E. Huettner, Co-Chairs

Functional recovery following spinal cord injury has been attributed to plasticity in local interneuron populations, which are able to create novel circuits that bypass the inhibitory lesion to activate pre-existing motor pathways. Cell replacement strategies and growth factor delivery platforms often attempt to enhance these natural mechanisms of regeneration. The work of this thesis sought to overcome the major obstacle to manipulating specific interneurons for therapy: an inability to obtain large, enriched interneuron subpopulations for drug screening and transplantation. We focused on the generation of V2a interneurons, which normally coordinate a wide range of locomotor tasks in both the spinal cord and respiratory centers of the hindbrain. They are an ipsilaterally projecting excitatory glutamatergic inteneuron population that is defined by expression of the homeodomain protein Chx10 during development. Using CRISPR/Cas9-mediated homologous recombineering, we generated a transgenic mouse embryonic stem cell (ESC) line that enabled us to positively select for Chx10+ ESC-derived V2a interneurons (ESC-V2as) by treating differentiated cells with puromycin. ESC-V2a cultures remained free of
proliferative cells, matured into normal glutamatergic neurons, and were capable of forming synapses onto motor neurons in adherent monolayer culture. For further in vitro investigation and transplantation studies, we cultured ESC-V2as and progenitor populations as spherical aggregates to generate a cellular microenvironment more closely related to that in vivo. We used this platform to test whether V2a IN axon growth could be stimulated in a dose-dependent manner by glial-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and platelet-derived neurotrophic factor (PDGF) and/or by co-culture with ESC-derived progenitor motor neurons (ESC-pMNs). Although ESC-V2as did not variably respond to the growth factors applied when cultured in isolation, co-culture with ESC-pMNs improved ESC-V2a neurite extension, survival, maturation, and electrophysiological activity. Application of optimized concentrations of GDNF and PDGF, which act specifically on ESC-pMNs but not ESC-V2a INs, enhanced these positive effects. Finally, we sought to determine the feasibility and efficacy of transplanting neural progenitor cells (NPCs) or ESC-pMNs enriched with ESC-V2as in a respiratory model of spinal cord injury. Despite the historic difficulty in transplanting post-mitotic neuronal populations, ESC-V2as were able to survive and migrate from injury site when co-transplanted with progenitors, and in the case of V2a/NPC groups, resulted in improved recovery of diaphragm activity compared to control groups. Taken together, this thesis establishes in vitro and in vivo methodologies to investigate possible mechanisms of V2a interneuron rewiring following spinal cord injury and a platform to optimize combinatorial treatment strategies moving forward.
Chapter 1

Introduction

1.1 Overview

Strategies to improve functional recovery after spinal cord injury (SCI) have been varied, encompassing gross training methods including rehabilitation, electrical stimulation, and hypoxia, as well as targeted interventions for local regeneration that seek to mitigate the inhibitory environment, to provide growth-promoting factors and substrates, and to replenish lost cell populations. Our lab has focused primarily on this latter set of strategies, working to bolster or enhance natural methods of plasticity about the injury site; in rare cases of spontaneous recovery, local neurons spared in the white matter around the lesion sprout collaterals that bypass the inhibitory scar and form functional relay circuits. The long-term goal of this research is to understand mechanisms of plasticity in the spinal cord after injury. Identifying cell types, biological factors, and pharmacological agents that are involved in these mechanisms will aid in the development of clinical interventions to improve patient outcomes.

Because of their role in central pattern generation (CPG), excitatory glutamatergic ventral interneurons (INs) are candidate populations to investigate for roles in functional rewiring events. There are two significant issues that hinder our ability to assess whether these populations are involved in regeneration after SCI: first, while the distinct developmental
transcription factor profiles that define ventral INs are increasingly well defined, a lack of mature identification markers has made study of endogenous populations in adults difficult. Second, any single subpopulation of ventral IN represents small fraction of cells in the spinal cord, complicating isolation for in vitro study or transplantation. Work in the Sakiyama-Elbert lab has sought to circumvent these issues by developing protocols to differentiate specific ventral IN populations from embryonic stem cells (ESCs). By genetically engineering transgenic ESC lines, large, homogenous populations of these INs can be used to evaluate therapeutic targets and for cell replacement strategies.

This thesis specifically focuses on creating a platform to study V2a INs, an ipsilaterally projecting, excitatory glutamatergic population of INs known to be actively involved in CPG networks in the lumbar and cervical spinal cord and respiratory centers of the hindbrain. The first aim of this work is to generate and characterize a selectable V2a-specific ESC line. The second aim is to develop a novel in vitro assay to study V2a INs in isolation or when co-cultured in order to determine whether the application of regenerative growth factors differentially improves their ability to mature and form functional synapses. The third aim is to assess the feasibility and efficacy of transplanting post-mitotic ESC-derived V2a INs into a respiratory model of SCI in rats. Together, these aims progress in vitro and in vivo methodologies to determine the role of V2a INs in regeneration after SCI.

In this chapter, I hope to convey the broad and interdisciplinary foundation of the thesis research herein, which has its basis in the confluence of regenerative medicine, developmental neurobiology, and stem cell engineering. The sections that follow describe the motivation for this work, the role of V2a INs and their potential for plasticity, how stem cells may be employed to
generate specific, clinically relevant INs for transplantation, and proposals for genetic strategies that could be used to demonstrate the potential of V2a INs to improve recovery after SCI.

1.2 Interneuron Plasticity after Spinal Cord Injury

The consequences of spinal cord injury (SCI) extend beyond the initial trauma, disrupting a variety of normal sensorimotor behaviors and having far reaching psychological and economic impacts to patients and healthcare systems. SCI disables more than 270,000 people in the United States with 12,500 new cases annually and estimated lifetime medical costs exceeding several million dollars per individual depending on the severity of functional loss (National Spinal Cord Injury Statistical Center 2014; DeVivo et al. 2011; Singh et al. 2014). SCI patients have lower life expectancies, lower employment rates, and lower chances for successful marriage compared to uninjured peers, trends which have not changed significantly in the last 40 years and which transcend international borders (National Spinal Cord Injury Statistical Center 2014; Singh et al. 2014).

Each traumatic SCI is unique and often occurs alongside multiple systemic injuries. This complicates both immediate and long-term management, thus making a one-treatment-fits-all strategy difficult. The primary mechanical trauma results in the immediate compression and disruption of axons and vasculature, triggering a secondary cascade of events including ischemia and excitotoxic chemical release, which exacerbate local cell death and significantly expand the injury site (Tator and Fehlings 1991). The rapid necrosis results in a cystic cavity that is infiltrated by inflammatory cells, microglia, fibroblasts and reactive astrocytes. These form a
dense, fibrous glial scar that expresses a multitude of inhibitory chemical cues and serves as a physical and chemical barrier that prevents regeneration across the lesion (Donnelly and Popovich 2008; Fawcett and Asher 1999; Schwab and Bartholdi 1996; Silver and Miller 2004). Wallerian degeneration, chronic demyelination, and muscle atrophy persist months or even years after the injury, limiting the potential for functional recovery over time (Totoiu and Keirstead 2005).

While spinal circuits were previously thought to be rigid with limited regenerative capacity after injury, research in the past few decades has uncovered remodeling in spared tissue that can result in spontaneous functional recovery. Regeneration of descending tracts from the brain is not necessarily or solely responsible for regained function; plasticity has been observed at various hierarchal levels in the central nervous system (CNS), including the cerebral cortex, limbic structures, corticospinal tracts (CST), propriospinal neurons (PNs), motor neurons (MNs) and segmental interneurons (INs) (Courtine et al. 2009; Isa and Nishimura 2014). Detour circuits formed by CST fibers, PNs, and local IN networks have been attributed to renewed innervation of intact central pattern generators (CPGs) (Filli and Schwab 2015; Flynn et al. 2011). Indeed, cell populations around the lesion are well placed to receive and respond to the host of pro-regenerative molecular cues that are upregulated after injury. These include molecules that are also expressed during development and signal intrinsic axon regeneration pathways as well as direct connectivity and synaptogenesis (Hollis 2015).

Spinal INs have been implicated in functional gains after SCI, but little is known about the cues or developmental origins of the INs involved in local rewiring events, in part because of the difficulty identifying specific contributions of different INs in vivo. Classically defined as
neurons whose cell bodies and projections are limited to the spinal cord, spinal INs are located in abundance throughout the length of the spinal cord and have diverse genetic, functional, and morphological properties that are continually being determined. The question is then, which IN populations are most important to target for regeneration? This section reviews the role of local INs in spontaneous recovery after SCI and proposes that specific ventral IN populations ought to be investigated for therapeutic use.

1.2.1 Rewiring Mechanisms for Spontaneous Recovery after SCI

Two seminal studies implicate INs in remodeling after SCI, and suggest two different rewiring mechanisms resulting in functional recovery (Figure 1.1). The first by Bareyre et al. was motivated by a desire to define the anatomical basis by which functional recovery occurs in the event of an incomplete SCI (Bareyre et al. 2004). Using a rat thoracic dorsal hemisection model, they found that severed CST axons sprout collaterals into spared gray matter rostral to the injury site, and that these collaterals project onto cervical INs within 3 weeks of the injury. Despite a reduction in the overall density of fibers 12 weeks post-injury, the remaining collaterals were those that had synapsed onto long INs with axons capable of arborizing onto MNs in the lumbar enlargement. Behavioral and electrophysiological tests confirmed that gain of function was caused by these new connections coupled with remodeling in the cortex (Bareyre et al. 2004). This study specifically implicates long INs that “bridge” the lesion site through spared tissue; while CST fibers also contact short INs at 3 weeks post-injury, these connections are significantly reduced by 12 weeks post-injury.

The alternative mechanism is a “relay” where short INs are responsible for recovery in
the absence of long distance regeneration. To demonstrate this phenomenon, Courtine et al. used an elegant temporally and spatially staggered lateral hemisection model, which severs all descending long tract axons by removing half of the projections at T12, and then later at T7. The mice were able to regain weight-bearing locomotor function within 10 weeks of each injury, which was not observed when the spinal cord was either fully transected or when the injuries occurred simultaneously. Using N-methyl-D-aspartate (NMDA) to ablate the local INs between the injuries caused a loss of functional recovery, which implies that local INs reorganize with time to form functional detour circuits around the lesion site (Courtine et al. 2008). This relay mechanism is often the basis for cell transplantation strategies involving neuronal populations; by replenishing appropriate populations at the lesion site, endogenous neurons may form functional relays with transplanted cells, thus reducing the distance required for regeneration. It is of note that the window of opportunity for this strategy may be limited by the survival of pertinent endogenous populations post-injury. A study by Conta et al. found that even though thoracic IN axons are severely damaged after a thoracic contusion injury, their cell bodies remain intact for up to 2 weeks (Conta and Stelzner 2004).

In a clinical setting, the bridging and relay mechanisms are not necessarily mutually exclusive; it is likely that, depending on the injury, sprouting collaterals and INs remodel according to the most stable arrangement. A goal of combinatorial therapies is to both encourage regeneration and remodeling across the lesions, as well as to provide substrate and/or cell populations that can mediate the formation of relay circuits. As it is unlikely that all spinal populations are equally involved in locomotor plasticity, an important consideration in the design of cell transplantation approaches is the type of cell to target. The two studies discussed above,
as well as subsequent studies, suggest that neurons of the lumbar spinal cord, which is the location of the mammalian walking CPG, are good IN populations to begin to investigate (Bareyre et al. 2004; Courtine et al. 2009; Courtine et al. 2008; Harkema 2008b; Lang et al. 2012).

Figure 1.1: Schematic showing the reorganization of IN circuits after temporally separated hemisections results in recovery of motor function as performed by Courtine et. al. in 2008. Lateral hemisections were performed at T12, with T7 injuries occurring either simultaneously or separated by 10 weeks. Significant functional recovery was only observed when INs located between the injury sites had time to remodel; excitotoxic ablation of local populations by NMDA abolished functional gains (Courtine et al. 2008). Figure adapted from (Flynn et al. 2011).

1.2.2 Ventral Interneurons in Locomotor Coordination

In the uninjured spinal cord, spinal INs network to form CPGs that provide input to MNs,
ultimately driving a multitude of rhythmic locomotor behaviors including breathing, left-right alternation, flexor extensor coordination, and other task-dependent patterned motions (Arber 2012; Goulding 2009; Kiehn 2006; Kiehn et al. 2010; Stepien and Arber 2008). For many decades, CPG INs were defined by their neurotransmitter profiles (glutamatergic, GABAergic, glycinerergic, serotonergic or cholinergic) paired with anatomical characteristics determined by tracing studies (laminae and segmental distributions, projections, and/or morphology). The emergence of better genetic tools, including lineage tracing and conditional knock-out animal models, has since allowed for the determination of functionally distinct IN populations that can be identified using developmental transcription factor expression profiles (Arber 2012).

**Figure 1.2:** Schematic showing the transcription factors expressed in the ventral half of the developing neural tube. The ventral-to-dorsal gradient of Shh and RA and relative positions of progenitor domains are shown on the left. Transcription factors expressed by interneuron domains (p1-p3) and pMN domains are shown in the middle. Transcription factors of mature, committed interneurons and MNs are on the far right (Adapted from (Brown et al. 2014b))

Many of the INs that make up CPG networks develop from the ventral neural tube during embryogenesis, and remain in the ventral horn of the adult spinal cord intermingled with MNs that project into the periphery. These ventral populations are derived from 5 cardinal progenitor
populations (p0, p1, p2, pMN, p3) that arise in response to various neurochemical gradients, most prominently retinoic acid (RA) from the somites and sonic hedgehog (Shh) from the floor plate (Briscoe and Ericson 2001) (Figure 1.2). There is a significant degree of subsequent diversification in mature neuronal populations; perhaps only a dozen genetically and functionally distinct IN populations have been characterized, with a high likelihood of additional subpopulation specification. Consequently, there are many holes in our understanding how spinal INs organize to generate rhythmic locomotion. Figures 1.2 and 1.3 summarize many of the known ventral progenitor and mature IN populations with corresponding transcription factor profiles, neurotransmitters, projections, and contributions to locomotor function.

Figure 1.3: Diagram describing the current understanding of ventral interneurons involved in spinal locomotion. This includes definitive expression markers, neurotransmitters, projections and functions of V0 INs (Griener et al. 2015; Lanuza et al. 2004; Moran-Rivard et al. 2001; Pierani et al. 2001; Talpalar et al. 2013; Zagoraíou et al. 2009), V1 INs (Alvarez et al. 2005; Benito-Gonzalez and Alvarez 2012; Britz et al. 2015; Eccles et al. 1954; Gosgnach et al. 2006; Renshaw 1946; Siembab et al. 2010; Zhang et al. 2014), V2 INs (Al-Mosawie et al. 2007; Azim et al. 2014; Britz et al. 2015; Crone et al. 2008; Crone et al. 2012; Crone et al. 2009; Dougherty and Kiehn 2010b; Dougherty et al. 2013; Okigawa et al. 2014; Panayi et al. 2010; Peng et al. 2007; Zhang et al. 2014) and V3 INs (Borowska et al. 2013; Briscoe et al. 1999; Zhang et al. 2008).
1.2.3 SCI Repair Strategies using Ventral Interneurons

Given the outsize role that ventral INs play in locomotor coordination, they represent candidate populations to target for interventions after SCI. Indeed, there is some evidence to suggest that ventral INs differentially improve locomotor recovery after transplantation compared to dorsal populations. In a relatively small study comparing respiratory recovery after the transplantation of either dorsal or ventral spinal tissue from fetal rats into a C2 hemisection model, animals with dorsal transplants exhibited a blunted phrenic response to respiratory challenge and, in some cases, failed to spontaneously recover ipsilateral phrenic MN activity as is typically the case for fetal-derived cell therapies. Animals treated with the ventral transplants also showed increased phrenic burst frequencies during hypoxia or hypocapnia compared to those with dorsal transplants, even though at baseline the burst frequency was demonstrably lower than in recovered animals treated with dorsal transplants (White et al. 2010). Other than this report, there has been little directed work investigating the role that ventral INs play in recovery after SCI.

There are several explanations as to the dearth of research in this area. As previously discussed, while the existence of CPGs was first demonstrated in the early 1960s (Wilson 1961), the identification unique IN populations has only accelerated in the last decade in the wake of better genetic tools and animal models (Goulding 2009). IN research has also primarily been undertaken by developmental neurobiologists; with the burgeoning interest in the formation of local IN relays as identified by Courtine et. al. in 2008, the SCI field is only now beginning to question how different IN populations may impact recovery. Even so, many of the published
transgenic mouse lines are temperamental or remain in private hands, and there is a scarcity of reliable antibodies with which to test for the presence of various INs in wild-type tissue, whether from the host spinal cord or grafted transplant. Stem cell transplants resulting in functional recovery after SCI differentiate into a wide variety of neurons, some of which are certain to be spinal IN populations, though they have been primarily identified by neurotransmitter expression as opposed to specific transcription factor profiles (Kobayashi et al. 2012; Nori et al. 2011). In the current environment, transplanting specific IN populations requires cell sorting of lineage-traced embryonic spinal cords, which is unreasonable given the financial and technical limitations of sorting post-mitotic tissue, of which only a small fraction comprises any single IN population.

The work in this thesis is part of an expansive effort by the Sakiyama-Elbert lab to use novel stem cell technologies and in vitro platforms as tools to overcome the limitations of IN research in the context of SCI as it stands today. The lab has concentrated specifically on excitatory populations, V0c, V2a, and V3 INs, which are directly involved in MN activity and, theoretically, ought to undergo significant plasticity for functional recovery. This thesis focuses specifically on the development of V2a INs as a therapeutic population.

1.3 V2a Interneurons

V2a INs constitute one of the most well characterized ventral IN populations, with a significant quantity of literature devoted to studying their development and contribution to locomotor coordination. This is in part because of the unusual number and reliability of animal
models and antibodies that have been developed by several groups, include the Sharma, Kiehn, and Jessell labs. Classically defined as INs that express Chx10, they are located along the entire rostrocaudal axis of the spinal cord and have varied functional roles that depend on their specific location. This section documents the specific roles that V2a INs occupy in the normal spinal cord and evidence that suggests that they undergo remodeling after injury.

1.3.1 V2a INs in Development

Complimentary gradients of Shh from the notochord and RA from the somites are required for the patterning of the ventral spinal cord, specifically resulting in spatiotemporal changes in seven necessary and sufficient homeobox genes: Pax7, Dbx1, Dbx2, Pax6, Nkx2.2, Nkx6.1 and Irx3. (Briscoe et al. 2000). Early during development, Shh represses Pax7 expression in ventral progenitor cells, which subsequently express Pax6 and Nkx2.2, two homeodomain proteins involved in the interpretation of the Shh gradient to specify cell fate. While initially Pax6 is expressed at all dorsoventral positions, Shh represses Pax6 in cells adjacent to the floor plate, initiating Nkx2.2 expression (Ericson et al. 1997a; Ericson et al. 1997b). V2 INs are generated from Pax6 progenitors, but knockout of Pax6 halves the V2 IN population as compared to the complete deletion of V1 INs. Transcription factor gradients that flank the p2 progenitor domain also impact V2 determination: the ventral limit of Dbx2 and dorsal limit of Nkx6.1 on the p1/p2 boundary and the ventral limit of Irx3 on the p2/pMN boundary. Misexpression studies demonstrate that coexpression of Nkx6.1 and Irx3 specifies p2 progenitors that give rise to V2 INs (Briscoe et al. 2000; Muhr et al. 2001).
The two main classes of V2 INs, V2a INs and V2b INs, arise from a common pool of Foxn4-expressing p2 progenitor cells. Foxn4 is a forkhead transcription factor that induces and modulates the mosaic expression of Neurog basic helix-loop-helix (bHLH), Mash1 (Ascl1) and delta-like 4 (Dll4), resulting in dual signaling cascades that cause V2a/V2b diversification (Del Barrio et al. 2007; Misra et al. 2014; Okigawa et al. 2014; Peng et al. 2007). Foxn4 and Ascl1 bind and activate Dll4 expression, but Neurog inhibits this activity, resulting in asymmetric Dll4 expression depending on the relative level of transcription factors available. Dll4 activates Notch1 and triggers a program involving BMP/TGF-β signaling to stimulate Gata2, Scl (Tal1), and Gata3 expression, culminating in the V2b population (Del Barrio et al. 2007; Li et al. 2005a; Misra et al. 2014; Muroyama et al. 2005). Complimentary progenitors that do not have activated Notch1, but are Dll4⁺ and Lhx3⁺ adopt the V2a fate with Chx10 expression (Del Barrio et al. 2007; Tanabe et al. 1998b).

For much of the past two decades, the genetic program for V2a INs was thought to be controlled by Lhx3, which also has a significant role in the development of MNs, located just ventral to the p2 domain (Peng et al. 2007; Sharma et al. 1998; Thaler et al. 2002). In the absence of other transcription factors, most notably MNR2, Hb9, and/or Isl1, there is ectopic expression V2a INs; misexpression of Lhx3 is also sufficient to induce the differentiation of Chx10⁺ V2a INs (Tanabe et al. 1998b; Thaler et al. 1999). Recent evidence suggests that Chx10, which has been used to identify V2a INs, but hadn’t been linked to roles in cell fate specification, is directly responsible for mediating the activity of Lhx3 to induce V2a IN fate (Clovis et al. 2016). Clovis et. al. demonstrate that by silencing Chx10 in vivo, Lhx3 failed to generate alternate V2a markers (Sox14, Shox2, Vglut2). Notably, over-expression of Chx10
alone is capable of driving V2a IN differentiation ectopically in the dorsal spinal cord and at the expense of MNs both *in vivo* and *in vitro*. Thus, while Lhx3 is required to induce endogenous Chx10 expression, Chx10 alone specifies V2a fate by repressing non-V2a genes, including MN genes. The latter is achieved by competitively binding to response elements that are typically activated by the Lhx3-IsIl complex required for MN specification (Clovis *et al.* 2016; Lee *et al.* 2012). Beyond its role as a transcriptional repressor, it is possible that Chx10 or its downstream transcription factors act as activators to upregulate other V2a IN genes (Clovis *et al.* 2016).

### 1.3.2 Organization of V2a INs

Over 80% of V2a INs are glutamatergic, expressing Vglut2, and though a small percentage may be glycinereric (1-5%), no GABAergic V2a INs have been identified (Al-Mosawie *et al.* 2007; Lundfald *et al.* 2007). V2a INs comprise approximately 30% of all ventral Vglut2⁺ neurons located in laminae VII, VIII, and X, thought to be the core region of the CPG (Crone *et al.* 2008; Kiehn and Kjaerulff 1998). Projection patterns for V2a INs in the spinal cord can be diverse, but most project locally to CPG and MN regions of the spinal cord. However, while some mammalian V2a INs may synapse onto MNs (Al-Mosawie *et al.* 2007; Dougherty and Kiehn 2010a), it is likely that they primarily project onto multiple classes of commissural interneurons (CINs), including V0 CINs that are Dbx1⁺/Evx1⁻ (Crone *et al.* 2008; Dougherty *et al.* 2013). Cervical V2a INs also make up a significant proportion of excitatory propriospinal INs that bridge the lateral reticular nucleus with motor regions of the spinal cord, therefore receiving both supraspinal and sensory input (Azim *et al.* 2014). In the hindbrain, V2a INs are only located within the medial reticular formation, and project into the ventrolateral medulla onto neurons of
the pre-Bötzinger complex (Bouvier et al. 2015; Bretzner and Brownstone 2013; Crone et al. 2012). V2a INs are a heterogeneous population both morphologically and genetically—they vary in size and span (Al-Mosawie et al. 2007), and at least one genetically identifiable subpopulation, a Chx10+/Shox2+ subset, has distinct behavioral roles (Dougherty et al. 2013). Other correlations between morphology, genetic identity, and locomotor function have yet to be made.

V2a INs are an exclusively ipsilaterally projecting population that shift from being both rostrally and caudally projecting at E13.5 to primarily caudally projecting later in development. The distribution of V2a INs varies throughout the developing E12.5 mouse spinal cord. V2b INs intermingle with a comparatively sparse V2a IN population in a wide central column at cervical levels. At thoracic levels, V2a and V2b INs become less intermingled, with V2b INs becoming more dorsally restricted while V2a INs remain in a narrower central column. V2a INs are more numerous and widely distributed along the dorsoventral axis at lumbar levels. There is somewhat of a differential rostrocaudal distribution of subsets of V2a INs, identified by the combinatorial presence of HNF-6, OC-2, OC-3, MafA, cMaf, Pou3F1, Prdm8, and/or Bhlhb5 (Francius et al. 2013). Although V2a INs have been found to settle primarily in laminae VII and project through the ventral cord in adult animals (Lundfald et al. 2007; Romer et al. 2016), little work has been done to determine whether there are significant changes in the rostrocaudal distribution of V2a INs in adults compared to the developing embryo in part due to difficulties tagging these populations.

Projection patterns, the population heterogeneity and several functional outputs are conserved in mice and zebrafish, where V2a INs are marked by the Chx10 homolog alx.
However, several key properties are as yet dissimilar. In zebrafish, the majority of V2a INs are last order-glutamatergic INs that synapse directly onto MNs, compared to a significant minority in rodents (Al-Mosawie et al. 2007; Kimura et al. 2006). V2a INs in zebrafish form monosynaptic connections onto MNs, and there are distinct microcircuit modules within V2a IN subclasses capable of activating slow, intermediate, or fast MNs depending on the type of locomotor activity engaged (Ampatzis et al. 2014; Kimura et al. 2006; Kimura et al. 2013; Kimura et al. 2008). A recent study demonstrated that V2a INs and MNs form functional ensembles through gap junctions, which allow MNs to actively, contribute to retrograde locomotor circuit recruitment (Song et al. 2016). Conversely, in mammals V2a INs project primarily onto other INs of the CPG and V2a IN coupling is sparse (Dougherty and Kiehn 2010a; Dougherty and Kiehn 2010b). Functionally distinct subsets of V2a INs have also been correlated to differences in birthdate in the developing zebrafish spinal cord, though these studies have not yielded unique genetic markers (Kimura et al. 2006) and have not been observed in rodents. While some of these findings may be replicated in mammals, it is apparent that the role of V2a INs is not fully conserved between species, probably due to the significant differences between the swimming and walking CPG circuits (Dougherty and Kiehn 2010b).

1.3.3 Role of V2a INs in the Mammalian Central Pattern Generator

Studies examining the specific functional roles of V2a INs in mammals have relied primarily on neonatal genetic ablation studies in fictive locomotion explant assays or ex vivo electrophysiological recordings from labeled slice cultures (Crone et al. 2008; Crone et al. 2012; Crone et al. 2009; Dougherty and Kiehn 2010a; Dougherty and Kiehn 2010b; Dougherty et al.
Like most ventral INs, V2a INs are required for respiratory function and Chx10-DTA mice die within a day of birth when bred on the commonly used C57BL/6 genetic background (Crone et al. 2008). By crossbreeding to the ICR background, a small proportion of heterozygous Chx10-DTA animals survive until adulthood for behavioral testing (Crone et al. 2009; Husch et al. 2015). Recent studies by the Jessell and Crone labs have taken advantage of virally-induced conditional ablations or chemogenetically-induced activation in adult Chx10-Cre animals (Azim et al. 2014; Romer et al. 2016). More sophisticated genetic models have also been developed by the Goulding lab to examine the role of V0 and V2b inhibitory INs, which may be translated to V2a INs in the future (Britz et al. 2015).

V2a IN activity in the lumbar spinal cord has been well characterized compared to activity elsewhere, likely because it is the location of the mammalian walking CPG. Genetic ablation of Chx10\(^+\) V2a INs disrupts speed-dependent left-right locomotor coordination and increases variability in locomotor burst activity (Crone et al. 2008; Crone et al. 2009). These irregularities in locomotor burst fidelity are caused in part by the Shox2\(^+\) V2a subpopulation (Dougherty et al. 2013). Flexor-extensor coordination, which is primarily influenced by inhibitory INs, remains unaffected by V2a ablation (Britz et al. 2015; Crone et al. 2008; Zhang et al. 2014). In adult moving animals, the lack of V2a INs is evidenced by apparent gait changes in response to speed—a trotting gait transitions to galloping as speed is increased, with an alteration in left-right limb coordination but not in forelimb-hindlimb coordination; these observations are consistent with drug-induced fictive locomotor recordings (Crone et al. 2009). Whole-cell patch recordings of the lumbar spinal cord reveal that V2a INs receive rhythmic excitatory input and have a wide variety of firing patterns, including tonic, delay, phasic,
chattering, and single-spike firing, that are rhythmogenic in response to drugs or ventral root-recorded motor activity (Dougherty and Kiehn 2010a; Zhong et al. 2010). Rhythmic firing or membrane fluctuations in Chx10+ cells also occur in response to induced flexor or extensor activity, as well as in response to serotonin (Dietz et al. 2012; Dougherty and Kiehn 2010a; Zhong et al. 2011). Distinct populations of V2a INs can be differentiated depending on these membrane and firing properties, and may play separate roles in the frequency-dependent activation of V2a INs, which occurs by compound recruitment of subthreshold INs (Zhong et al. 2010; Zhong et al. 2011). It is of note that adult V2a INs undergo significant postnatal development in their intrinsic electrophysiological properties and firing patterns; comparing neonates to mature P43 mice, the Harris-Warrick group found age-dependent increases in excitability as well as the emergence of serotonin-dependent membrane potential bistability (Husch et al. 2015). These observations demonstrate the limitations of neonatal fictive locomotor assays.

V2a INs in the cervical spinal cord and hindbrain contribute to different locomotor pathways from those in the lumbar cord. More than 30% of V2a INs in the cervical spinal cord comprise of excitatory propriospinal INs, which project caudally to MNs responsible for forelimb activation as well as rostrally to the lateral reticular nucleus. These propriospinal V2a INs span from C1 to T1, and when selectively ablated or photostimulated, they perturb the fidelity of task-related skilled reaching behaviors, but not grasping (Azim et al. 2014). They are also central to an internal copy pathway, which allows for rapid modulation of motor output by helping to match premotor commands to internal reports. Propriospinal V2a INs recruit neurons of the lateral reticular nucleus, which likely project to deep cerebellar nuclei that in turn recruit
reticulospinal neurons, MNs, and additional propriospinal neurons, forming an internal feedback loop (Azim et al. 2014).

V2a INs of the hindbrain are primarily recruited into breathing circuits; they project directly from the medial reticulus to the pre-Bötzinger complex, which is responsible respiratory rhythm generation. Ablation of Chx10+ cells in neonates causes irregular breathing at a reduced frequency; as these mice mature, breathing becomes more regular, though the frequency remains reduced (Crone et al. 2012). Interestingly, the genetic background of the mutant plays a significant role in the extent of the breathing phenotype and therefore the viability of offspring (Crone et al. 2008; Crone et al. 2012; Crone et al. 2009). Slice preparations show that the absence of V2a INs in the medulla compromises the central respiratory rhythm generator, but activity can be rescued by bath application of compounds that increase breathing frequency (Crone et al. 2012). When selectively activated using designer drugs, some combination of cervical spinal and reticulospinal V2a INs are sufficient to drive inspiratory accessory respiratory muscle (ARM) activity in adults, which could have implications for respiratory compensation and degeneration in disease and trauma (Romer et al. 2016). V2a INs also constitute a significant proportion of glutamatergic reticulospinal pathways, and they have been shown to both mediate descending locomotor commands, as well as “stop” commands that may control episodic locomotion (Bouvier et al. 2015; Bretzner and Brownstone 2013).

These studies demonstrate that V2a INs contribute to distinct microcircuits throughout the spinal cord that are able to encode specific locomotor behaviors, which in many cases function independent of cerebellar input. However, V2a INs in of themselves are not rhythm generators, nor does their absence in the CPG eradicate a locomotor behavior that cannot in some
way be compensated for by other INs. Genetically discrete subpopulations have yet to be linked
to functional roles that are differentially distributed along the rostrocaudal axis. Despite these
shortcomings as an ideal therapeutic population, entrenched as they are in multiple locomotor
pathways, V2a INs remain one of the best gateways into affecting spinal plasticity after injury.

1.3.4 V2a INs in Spinal Cord Injury

Following incomplete SCI in rodents, spontaneous recovery of locomotion may be
achieved through rewiring of newly sprouted descending tracts with local IN populations
(Bareyre et al. 2004; Courtine et al. 2008; Filli and Schwab 2015; Isa and Nishimura 2014). A
few recent studies suggest that endogenous and transplanted V2a INs may play a role in these
rewiring events. The Harris-Warrick group directly investigated the effect of SCI on intrinsic
V2a qualities, demonstrating that neuromodulation caused by serotonin changes significantly in
V2a INs after SCI using Chx10-eGFP transgenic mice (Husch et al. 2012). Specifically, there is
a 100 to 1000-fold increase in the serotonin sensitivity, but no change in membrane excitability,
which contrasts with the hyperexcitability observed in MNs (Husch et al. 2012; Murray et al.
2010). It is probable that this type of selective plasticity occurs in other IN populations as well,
and could influence how these neurons learn to interpret and propagate new signals to pre-
existing CPG circuits. Another study showed that a single injection of docosahexaenoic acid
after SCI promotes sprouting in uninjured corticospinal and serotonergic fibers, which make
contacts on caudal V2a INs in both a rat cervical hemisection model as well as in a mouse
pyramidotomy model of SCI. Functional recovery could be correlated to the quantity of fiber
contacts onto Chx10+ V2a INs, which was significantly increased with their treatment (Liu et al.
While this study was biased in its exclusive examination of V2a INs and MNs and does not show that V2a INs are specifically responsible for the observed behavioral gains, it demonstrates that regenerating fibers do engage with intact V2a IN circuits. In zebrafish, large numbers of V2 INs are actually generated near the SCI lesion site, possibly by ependymal cells derived from the p2 domain during development; this is in contrast to comparatively low production of more dorsal populations (Kuscha et al. 2012). As previously discussed, many disparities have been observed between V2a INs in mammals and zebrafish, but the concept of differential IN differentiation from ependymal cells certainly has merit in light of other studies showing that ventral IN populations are a more efficacious transplantable population (White et al. 2010). A study by the Lane lab showed that after transplantation of fetal spinal cord tissue into a rat C2-C3 cervical contusion model of SCI, Chx10\(^+\) V2a INs were detectable after several weeks in animals that had recovered breathing function. Transplantation of cultured NSCs into the same model did not yield detectable V2a INs nor the same functional recovery (unpublished correspondence, (Zholudeva et al. 2015)). Again, while the presence of V2a INs are simply correlative to behavioral gains, not causative, these experiments lend credence to the idea that cardinal IN populations involved in CPG circuitry likely play a role in remodeling after SCI. A more recent unpublished study by the Lane lab has shown more direct evidence. Using transgenic Chx10-Cre animals, they demonstrate that while V2a INs do not normally participate in the phrenic motor pathways, after SCI, transynaptic tracing of phrenic pools marks these populations, which implies recruitment into newly reorganized circuits.
Figure 1.4: Transplantation of stem cell-derived spinal populations into the SCI lesion has multiple benefits. Neural stem cells (NSCs) can differentiate into mature neuronal and glial populations to replace lost or damaged cells. These populations can serve as relay circuits between intact tissue across the lesion, and thus promote functional improvements as determined by electrophysiological and behavioral means. Stem cell-derived oligodendrocytes and astrocytes can remyelinate damaged axons and provide scaffolding for regenerating axons, as well provide trophic support through the secretion of growth factors.

1.4 Stem Cell Therapies for Spinal Cord Injury

Although early surgical interventions for SCI focus on reducing the amount of damage done by secondary processes and stabilizing the spinal cord, most treatments emphasize neuroprotection, neuroregeneration, and rehabilitation. Cell-based therapies have gained popularity as a research focus because they can provide multiple benefits, including replenishment of lost cell types, scaffolding for axon regeneration, and the delivery of immuno-
modulatory, neurotrophic and anti-inhibitory factors. Neural progenitor cells, mesenchymal stromal cells, olfactory ensheathing glia, Schwann cells, and various pluripotent stem cells are among those currently investigated for their utility after SCI. Here I focus specifically on stem cells and strategies that have been used to differentiate and deliver them after SCI.

1.4.1 Stem Cell Sources

Multipotent Stem Cells

Two types of multipotent stem cells, unspecialized cells capable of differentiating into a discrete population of specific cell types of the same germ layer, are primarily investigated for treatment after SCI: MSCs and neural stem cells (NSCs) (Figure 1.5). MSCs are appealing clinically because they can differentiate into many non-hematopoietic lineages, but are also easily isolated from patient bone marrow, thus allowing for autologous transplantation. However, several studies have demonstrated that their utility is confined to trophic and immunomodulatory effects after SCI in both animals and patients (Hofstetter et al. 2002; Parr et al. 2007; Tetzlaff et al. 2011; Urdzikova et al. 2014). High variability between studies, limited functional recovery, poor engraftment, and questionable neuronal differentiation in vivo limit the use of MSCs for cell replacement (Tetzlaff et al. 2011).

NSCs have been widely studied for transplantation after SCI because their maturation is restricted to glial and neuronal subtypes, thus reducing tumorigenicity while replenishing lost cells, aiding in remyelination and trophic factor secretion, and promoting axon regeneration. NSCs can be harvested from either adult or fetal spinal cord tissue and expanded as neurospheres in the presence of growth factors, including epidermal growth factor (EGF) and/or basic
fibroblast growth factor (FGF2), prior to transplantation (Brewer and Torricelli 2007; Shihabuddin et al. 1997; Uchida et al. 2000; Weiss et al. 1996) (Figure 1.5). Fetal NSCs are generally heterogeneous, containing a mixture of neuronal and glial restricted progenitor cells, as well as self-renewing stem cells (Tetzlaff et al. 2011); in adults, ependymal cells along the central canal are NSCs that respond dramatically after SCI and constitute an endogenous source of stem cells to target (Barnabe-Heider et al. 2010; Johansson et al. 1999; McTigue et al. 2001; Weiss et al. 1996; Yang et al. 2006). Because NSCs can retain their positional identity through in vitro expansion, anatomical origin is an important consideration for cell replacement therapy and can be exploited to maximize integration into host spinal circuits (Hitoshi et al. 2002; Philippidou and Dasen 2013).

Functional recovery after NSC transplantation has been observed in a variety of animal models and can be enhanced by co-treatments with trophic factors (Tetzlaff et al. 2011). Though NSCs are capable of differentiating into all CNS types, both endogenous and transplanted NSCs in the spinal cord overwhelmingly become astrocytes and oligodendrocytes, with variable neuronal differentiation (Barnabe-Heider et al. 2010; Cao et al. 2001; Karimi-Abdolrezaee et al. 2006; Kriegstein and Alvarez-Buylla 2009; Parr et al. 2008). Furthermore, despite their many positive attributes, NSCs cannot be used for autologous transplantation and may be excluded from clinical use by contentions deriving them from fetal or post-mortem patient tissue. To circumvent this issue, many labs generate NSCs from pluripotent stem cells or directly reprogram them from somatic cells, such as fibroblasts.

**Pluripotent Stem Cells**
Pluripotent stem cells (PSCs) are characterized by their ability to replicate indefinitely while maintaining the ability to differentiate into specialized cell lineages from all three embryonic germ layers. Embryonic stem cells (ESCs) derived from the inner cell mass of pre-implantation blastocysts were the first isolated, differentiated, and proposed for use in cell therapy after SCI (Evans and Kaufman 1981; McDonald et al. 1999; Thomson et al. 1998)(Figure 1.5). In the absence of factors that maintain pluripotency, such as leukemia inhibitory factor (LIF), FGFs, or rho-associated protein kinase (ROCK) inhibitors (Smith et al. 1988; Watanabe et al. 2007; Williams et al. 1988), ESCs spontaneously differentiate; many strategies have been developed to preferentially induce differentiation along spinal neural lineages and even specify a positional identity along the neuraxis, discussed below. While several studies have shown benefits associated with ESC-derived neuronal transplants (Hatami et al. 2009; Johnson et al. 2010b), ethical concerns and potential side effects including teratoma formation caused by the presence of undifferentiated ESCs prevent clinical application.

Induced pluripotent stem cells (iPSCs) have become an appealing alternative to ESCs and allow for autologous transplantation of patient-derived cells. By reprogramming somatic cells with a defined set of transcription factors, the Yamanaka factors, adult mouse and human cells revert to a pluripotent state, thus alleviating ethical impediments caused by the destruction of embryos (Takahashi et al. 2007; Takahashi and Yamanaka 2006) (Figure 1.5). Differentiation strategies initially developed for ESCs have been translated to iPSCs. However, similar to ESCs, transplantation of iPSCs retains a high risk of immune rejection and tumorgenesis, the latter of which can be compounded by virally overexpressing oncogenic transcription factors for iPSC generation. Safer, non-viral methods have been developed, but spontaneous reversion to a
pluripotent state is a unique concern for iPSC-derived cells and contributes to the tumorigenic potential (Chen et al. 2013a; Saric et al. 2008). Considerations including the mechanism of reprogramming, the cell source, and the differentiation process are important to weigh in designing therapeutic strategies involving pluripotent stem cells (Khazaei et al. 2014).
Figure 1.5: Stem cell sources. There are several sources of multipotent (left) and pluripotent (right) stem cells currently used for SCI. Neural stem cells (NSCs) can be derived from fetal or adult tissue, and are capable of differentiating into neurons, oligodendrocytes, and astrocytes. While not typically considered stem cells, glial-restricted precursors (GRPs) are a commonly studied, tri-potent population that can be isolated from neural stem cells or fetal tissue directly. GRPs differentiate into oligodendrocyte progenitor cells and two types of astrocytes. Mesenchymal stromal cells (MSCs) are an appealing population clinically because they can be isolated from adult bone marrow or peripheral blood; however, while they are capable of differentiating into a wide variety of cells types, the efficacy of neuronal differentiation is a specific concern for SCI treatment. Embryonic stem cells (ESCs) are a pluripotent population, which can give rise to cell types from all three germ layers; however, because they are derived from the inner cell mass of early blastocysts, ethical considerations limit their clinical potential. Induced pluripotent stem cells (iPSCs) can be generated from adult somatic cells (fibroblasts, melanocytes, cord or peripheral blood cells, adipose stem cells, etc.) by several different reprogramming methods using the Yamanaka factors (c-Myc, Sox2, Oct4, Klf2). While induction and reprogramming efficiencies remain a concern, iPSCs represent an autologous, patient-specific population that has significant clinical potential as the field progresses.

1.4.2 Directing Neural Fate

Cell-Cell Interactions

Neural induction is a default pathway for PSCs, but differentiation efficiency varies depending on both intrinsic cell line qualities, as well as the extracellular environment (Bain et al. 1995; Hu et al. 2010; Munoz-Sanjuan and Brivanlou 2002) (Figure 1.6). Spatial cues play an important role in cell fate determination and can dramatically alter the efficiency of neural induction. Two dimensional (2D) adherent monolayer cultures or co-culture with feeder cells have been used with varying success, though the use of micropatterned surfaces or manual selection of spontaneous rosette structures can help direct neuronal morphogenesis (Knight et al. 2015; Zhang et al. 2001). Allowing cultures to grow in three dimensions (3D), instead of in 2D, generates environmental conditions that more closely mimic those found in vivo, and can
consequently enhance neuronal differentiation. Non-adherent cultures that allow PSCs to aggregate into embryoid bodies (EBs) have traditionally been used, since EBs develop similarly to the pre-gastrulation embryo and are primed for application of patterning factors that can direct more specific cell types (Weitzer 2006). A problem with EBs is that they are heterogeneous and disorganized, which frequently results in variable neural induction. To improve differentiation consistency, several methods have been developed to control EB dimensions beyond static suspension culture, including the hanging drop method, cell encapsulation, microwell or microfabrication methods, and the use of bioreactors (Rungarunlert et al. 2009).

New 3D culture systems are now gaining traction. PSC-derived organoid cultures, evolved from EBs, have become appealing for in vitro disease modeling and as a source for complex cell populations (Huch and Koo 2015; Lancaster and Knoblich 2014). Several protocols exist to generate organoids for individual brain regions or heterogeneous cerebral organoids containing multiple regions (Lancaster and Knoblich 2014). The reliance on random structure formation can be avoided by using defined 3D scaffolds. Using Matrigel or a synthetic matrix separately, the Tanaka group was able to generate neuroepithelial cysts from single mouse ESCs that undergo classic dorsoventral patterning when in the presence of retinoic acid (RA) (Meinhardt et al. 2014). These studies demonstrate not only the feasibility of creating complex, organized spinal tissue, but also how bioengineered 3D scaffolds can be used to finely control the stem cell microenvironment. Such scaffolds can also be designed as transplantation vehicles for SCI treatment (Murphy and Atala 2014; Willerth and Sakiyama-Elbert 2008). We have done significant work optimizing neural differentiation within fibrin gels that have been modified for controlled release of growth factors or co-delivery of anti-inhibitory molecules. After
transplantation, these combinatorial treatments are able to improve cell survival, differentiation, and neurite growth at the host-graft interface (Johnson et al. 2010b; McCreedy et al. 2014b; Wilems et al. 2015; Willerth et al. 2006; Willerth et al. 2007; Willerth et al. 2008).

**Figure 1.6: Variables in stem cell culture.** Culture conditions have an enormous impact on neuronal induction efficiency, but the mechanism by which these variables influence cell fate determination is poorly understood. Both 2D and 3D culture formats are used, the most common of which are embryoid bodies, biomaterials scaffolds, adherent culture, and co-culture with feeder cells. Other variables include the density and heterogeneity of cells, the composition of media and the extracellular environment, including the induction substrate, temperature, pH, and CO$_2$ concentration. The concentration, duration, and type of morphogen used are the experimental conditions most commonly altered to direct differentiation to a specific lineage.

**Mimicking Development to Drive Differentiation**

The traditional method of converting PSCs to CNS cells involves recapitulating the developmental environment of the neural tube via exposure to growth factors and morphogens (Figure 1.7). Induction efficiency and lineage specification can be adjusted by modulating the concentration and exposure duration of these factors. Early induction protocols called for the use
of harvested or recombinant protein, but a more sophisticated understanding of the signaling, transcriptional, and epigenetic cues responsible for cell fate specification in recent years has enabled the use of small molecules to direct differentiation in lieu of proteins, which can be expensive and labile.

**Neural Induction**

Neural induction and the generation of nestin+ NSCs is the first step towards differentiating specific spinal cell types from PSCs. While the spinal cord is a caudal CNS structure, the neural cells from which it is formed initially acquire a rostral character through a combination of transforming growth factor (TGF)-β, bone morphogenetic protein (BMP), FGF, and Wnt signaling (Munoz-Sanjuan and Brivanlou 2002; Wilson and Edlund 2001). Forming EBs results in spontaneous NSC production, but as previously discussed, the process can give variable neuronal yields (Bain et al. 1995). Taking cues from development, several groups have efficiently induced a neural fate by inhibiting TGF-β/Activin/Nodal and BMP signaling pathways *in vitro* using recombinant inhibitors or small molecule antagonists (Chambers et al. 2009; Chambers et al. 2012; Patani et al. 2009; Smith et al. 2008). EGF and FGF2 have been used to isolate NSCs from primary tissue, but can also promote survival and induction of NSCs from PSCs (Chambers et al. 2012; Joannides et al. 2007; Okabe et al. 1996; Reubinoff et al. 2001; Shihabuddin et al. 1997). The timing of growth factor exposure is important; after NSCs have developed, inhibition of FGF2 *in vitro* aids in the differentiation of NSCs into neurons and thus improves differentiation efficiency (Chambers et al. 2012; Joannides et al. 2007).
Caudalization with Retinoic Acid

Endogenous neural progenitors acquire a spinal identity in response to caudalizing signals, predominantly RA, which is involved in both anteroposterior and dorsoventral patterning of the neural tube (Durston et al. 1998; Muhr et al. 1999). RA inhibits FGF signaling and triggers differentiation in the neuroepithelium (Diez del Corral et al. 2003; Novitch et al. 2003). Treatment of ESCs with RA was among the first efforts to obtain spinal neurons; however, when used for prolonged durations, RA functions as a differentiation agent that significantly impacts neuronal subtype specification in the ventral spinal cord (Bain et al. 1995; Jessell 2000; Maden 2007; Wilson and Maden 2005). It is of note that RA serves many neuroprotective and neuroregenerative roles in the adult CNS and could have value for SCI therapy beyond its use in neural induction (Maden 2007).

Growth Factor-Mediated Patterning

A combination of factors released from mesodermal structures around the neural tube is responsible for neuronal differentiation: RA from the somites, sonic hedgehog (Shh) from the notochord, BMPs, EGFs, and Wnts from the ectoderm and roof plate, and FGFs from the posterior of the neural tube (Jessell 2000; Lee and Jessell 1999). While research parsing endogenous differentiation pathways has been ongoing for more than two decades, new genes and transcription factors are continually added to the current models as spinal neuron subtypes are being defined. The signaling involved in cell fate specification is synergistic, time-dependent, and concentration-dependent. The sensitivity of differentiation means slight changes to PSC induction protocols can significantly alter the PSC-derived neuronal population.
distribution, but also allows for optimization of specific subpopulations.

In the ventral spinal cord, RA and Shh work together to induce four classes of ventral IN progenitors (p0-p3), which give rise to various ventral INs, as well as a progenitor MN (pMN) domain, which gives rise to MNs, astrocytes, and oligodendrocytes (Briscoe and Ericson 2001; Wilson and Maden 2005). These neuronal populations contribute to central pattern generator networks in the spinal cord that are critical for normal motor function and coordination (Arber 2012). A significant body of work by the Jessell lab has demonstrated the importance of Shh-dependent, differential homeobox transcription factor expression in generating the discrete ventral progenitor domains, as well as specification of neuronal subtypes (Briscoe and Ericson 2001). RA and Shh are necessary and sufficient to induce ventral spinal differentiation from PSCs; by modulating the relative concentrations and durations of signaling factors, they and others have been able to optimize induction protocols to enrich for specific ventral populations (Brown et al. 2014b; Dessaud et al. 2010; Kim et al. 2009; Li et al. 2005b; McCreedy et al. 2012; Okada et al. 2004; Wichterle et al. 2002; Xu and Sakiyama-Elbert 2015b).

Little work has been done to develop PSC-derived populations from the dorsal spinal cord, in part because the molecular mechanisms involved in diversification are not as well established as those in the ventral cord (Helms and Johnson 2003; Lee and Jessell 1999). Dorsal INs are primarily associated with sensory circuits, though dI3 and dI6 INs have been implicated in motor coordination (Bui et al. 2013; Dyck et al. 2012). There are six classes of early-born dorsal INs (dI1-dI6) as well as two late-born ones (dIL A, dIL B). These domains can be further subdivided by their dependence on BMP signaling from the roof-plate for development: dependent Class A (dI1-dI3) and independent Class B (dI4-dI6, dIL A/B). Similar to Shh signaling
in the ventral spinal cord, gradients of BMP activity contribute to the formation of discrete homeobox transcription factor expression domains that give rise to Class A progenitor cells (Timmer et al. 2002). Treatment of EBs with RA alone induces differentiation of dorsal Class B INs and V0 and V1 INs, which are less dependent on roof plate and floor plate signaling for development (Kim et al. 2009). Wnt, FGF, and EGF signaling are important for initial neural crest development, but their roles in dorsal IN diversification are less well understood (Helms and Johnson 2003; Lee et al. 2000; Lee and Jessell 1999; Muller et al. 2002). At least one group has shown that a combination of these developmental factors directs dorsal IN differentiation in mouse embryonic stem cells (Murashov et al. 2005). Others have bypassed neural induction entirely and used transcriptional programming to obtain sensory neurons from fibroblasts (Blanchard et al. 2015).

Refining Inductions for Subtype Specification

There are many subpopulations of neurons and glia beyond the cardinal classes defined during spinal cord development. While spinal oligodendrocytes and astrocytes arise from the pMN domain, which requires RA/Shh signaling, they also occur elsewhere in Shh independent ways and can require the addition of FGFs, BMPs, tumor necrosis factors (TNF), interleukins (ILs), platelet-derived growth factor (PDGF) and/or ciliary neurotrophic factor (CNTF) signaling to direct their differentiation in vitro and in vivo (Benveniste et al. 2005; Douvaras and Fossati 2015; Goldman and Kuypers 2015; Kencik and Zhang 2011; Nistor et al. 2005; Roybon et al. 2013). Many IN populations have not yet been classified by unique transcription factor profiles, and the populations that have been characterized are certain to be even more subdivided. As
additional spinal subtypes and their differentiation mechanisms are identified, it will be important to translate those to optimize PSC induction protocols. For example, Notch signaling acts as a transcriptional switch between inhibitory and excitatory IN subpopulations in both dIL and V2 INs, and possibly contributes to diversification elsewhere in the spinal cord (Del Barrio et al. 2007; Mizuguchi et al. 2006). By adding a Notch inhibitor to an RA/Shh induction, V2a INs can be selectively enriched in PSC-derived cultures (Brown et al. 2014b).

There has recently been interest in developing PSC-derived populations that not only comprise of defined neuronal subtypes, but also have a defined positional identity, as these are important for migration and integration into host motor circuits (Philippidou and Dasen 2013). Much of this work has been done in MNs, which have genetically defined subtypes that are anatomically and functionally distinct. Subtle changes to the induction can alter Hox signaling and thus positional identity, including the type of signaling agent used (inhibitor versus recombinant protein), the exposure time and sequence of induction factors, and the addition factors that stimulate FGF and Wnt/β-catenin signaling (Davis-Dusenbery et al. 2014; Lippmann et al. 2015; Maden 2007; Patani et al. 2009; Patani et al. 2011; Peljto et al. 2010). Stem cell therapies that match host cell populations as closely as possible will provide the best opportunity for success.
Figure 1.7: Directing differentiation of pluripotent stem cells is traditionally achieved by triggering the signaling cascades responsible for cell fate determination. Neural induction from pluripotent cells typically begins with conversion into a neural stem cell (NSC), which may involve stimulating EGF and FGF2 signaling and inhibiting BMP and TGF-β/Activin/Nodal signaling. Subsequent exposure with RA caudalizes the NSCs and ensures a spinal identity. A complex interplay of signaling events is responsible for the dorsoventral patterning of the spinal cord, made more opaque by the importance of exposure duration and cell-cell signaling events. In the developing spinal cord, Shh from the notochord and floor plate interact with RA to induce differentiation of ventral progenitor cells (p0-p3, pMN), which mature into MNs, oligodendrocytes, astrocytes, and a variety of ventral IN populations. A combination of BMP, Wnt, FGF, and TGF-β signaling induces differentiation of the dorsal progenitor populations (dI1-dI6), but the precise mechanisms are poorly understood. Some specific factors that influence progenitor subspecialization have been determined experimentally in vitro, especially with regards to glial-restricted progenitor cells (GRPs), but for most IN populations, these are unknown.

**Transcriptional Reprogramming**

Driving differentiation of PSCs in a chemically defined environment has limitations, especially in human cells where induction and maturation can take months. Despite efforts to generate specific spinal cell types, the co-dependence in signaling involved in neuronal diversification necessarily leads to heterogeneity in cultures that use common induction factors. Overexpressing transcription factors directly responsible for neuronal subtype specification is a way to obtain more homogeneous cultures while minimizing induction time. Overexpression of just Ngn2 or NeuroD1 in PSCs results in efficient conversion into functionally active neurons (Zhang et al. 2013). Precise manipulation of transcription factor activation is important for cell type specificity; spinal MNs can be generated using a combination of Ngn2, Isl1, and Lhx3 (LIN factors), but replacement of Lhx3 with Phox2a yields cranial MNs (Hester et al. 2011; Mazzoni et al. 2013).
Direct reprogramming from one somatic cell type to another has become possible, bypassing an intermediate pluripotent state. Avoiding iPSC generation can potentially reduce the time to generate a specific cell type and minimizes the risk of teratoma formation upon transplantation. Several combinations of transcription factors convert fibroblasts to directly reprogrammed NSCs (iNSCs), though it is possible to generate tripotent iNSCs that can become neurons, astrocytes and oligodendrocytes by expressing just Sox2 or Oct4 (Han et al. 2012; Kim et al. 2011; Lujan et al. 2012; Mitchell et al. 2014; Ring et al. 2012). Vierbuchen et al. demonstrated that expression of Brn2 (Pou3f2), Ascl1, and Myt1l (BAM factors) were sufficient to convert mouse fibroblasts to functionally mature, excitatory neurons (iNs) (Vierbuchen et al. 2010). A variety of distinct neuronal and glial populations have since been generated from both mouse and human somatic cells, including dopaminergic neurons, sensory neurons, MNs, oligodendrocyte precursor cells (OPCs), and astrocytes. (Blanchard et al. 2015; Caiazzo et al. 2011; Caiazzo et al. 2015; Marro and Yang 2014; Najm et al. 2013; Son et al. 2011; Yang et al. 2013a). Of interest for SCI, astrocytes have been directly converted to iNSCs and functional neurons both in vitro and in vivo (Corti et al. 2012; Guo et al. 2014).

Very low neuronal conversion efficiencies—hovering near 1% using certain protocols—remain a hurdle facing direct reprogramming as a clinically relevant method for cell replacement. Depending on the protocol efficiency, mitotic cells, such as fibroblasts, must be significantly expanded in order to obtain a sufficient quantity of desirable cells, and then purified prior to transplantation, which can be expensive and time consuming. The sub-optimal conversion rates may not induce therapeutic outcomes that overcome the risks associated with reprogramming. Several ways to enhance the reprogramming process are in development. For example,
microRNAs can behave synergistically with the BAM factors to more efficiently convert iNs (Ambasudhan et al. 2011; Yoo et al. 2011). iPSC reprogramming can be optimized by blocking DNA and histone methylation or modulating chromatin structure to open transcriptional binding sites (Huangfu et al. 2008; Lin et al. 2009; Luna-Zurita and Bruneau 2013; Tso and McKinnon 2015). Direct reprogramming has recently been achieved by delivering small molecules (Hu et al. 2015; Li et al. 2015); this is clinically relevant, as it avoids potentially hazardous viral transductions and presents the opportunity to bioengineer delivery systems to convert endogenous cells into cell types of interest. This approach may be more viable to replenish neurons by transcriptional reprogramming, since transplanted iNSCs suffer from poor engraftment (Hong et al. 2014).

1.4.3 Transplantation Outcomes of Stem Cell Derived Spinal Populations

Considerations for Transplantation

The optimal window for cell therapy is generally thought to be within two to four weeks of the initial trauma, when the acute phase of the secondary injury has abated and while severed axons and surviving IN populations are still capable of responding to the host of axon guidance molecules and neurotrophins upregulated after SCI (Bareyre et al. 2004; Conta and Stelzner 2004; Coumans et al. 2001; Courtine et al. 2008; Hayashi et al. 2000; Hollis 2015; Lang et al. 2012; Schwab and Bartholdi 1996; Widenfalk et al. 2001). Multiple groups have observed that delayed transplantation into a more chronic injury is beneficial compared to acute treatment (Coumans et al. 2001; Karimi-Abdolrezaee et al. 2006), possibly because of alterations in the inflammatory response that allow for better cell survival in both endogenous and transplanted
populations (David et al. 2012; Donnelly and Popovich 2008; Moreno-Manzano et al. 2009; Rolls et al. 2009). Beyond that timeframe, the glial scar stabilizes and the upregulation of pro-regenerative factors attenuates, thus reducing the potential for regeneration (Cregg et al. 2014; Satake et al. 2000; Silver and Miller 2004; Widenfalk et al. 2001). The intended mechanism by which the transplant stimulates recovery is an important consideration when choosing both the time of intervention and the specific cell population for transplantation (Bradbury and McMahon 2006). For example, the replenishment of neuronal populations is critical to take advantage of functional relay mechanisms (Bareyre et al. 2004; Courtine et al. 2008), but post-mitotic neurons suffer upon transplantation, so using plastic progenitor populations may be a more reasonable approach. At the same time, inhibitory proteoglycans and trophic factors secreted by reactive astrocytes have differential effects on stem cell differentiation and survival, which may significantly alter the composition of the transplant. (Rolls et al. 2009; Silver and Miller 2004). Transplanting glia, which have roles in trophic support or immunomodulation, may mitigate some inhibition from the lesion (Liddelow and Hoyer 2016; Zeis et al. 2015). Anecdotal evidence from Asterias’ clinical trial suggests that, even after cessation of immunosuppression, human ESC-derived OPCs that successfully integrated into host networks can survive years after transplantation (Asterias 2016).

**PSC-derived NSCs**

PSC-derived NSC transplants have numerous neuroprotective and regenerative benefits beyond cell replacement including immunomodulation, the secretion of neurotrophic factors to enhance endogenous cell survival, axon regeneration, and remyelination initiated by NSC-
derived oligodendrocytes. Most studies demonstrate that transplants are capable of differentiating into neurons, astrocytes, and oligodendrocytes in vivo, though the ratios may differ depending on the method of NSC differentiation, the SCI model, and time point of investigation (Kobayashi et al. 2012; Nori et al. 2011). The extent of functional recovery also varies between studies. Significant recovery has been observed after human iPSC-derived NSC transplantation with no tumorigenicity and integration into host neural circuits, prompting consideration for clinical trials (Kobayashi et al. 2012; Nori et al. 2011). However, others have shown only marginal behavioral improvements despite good cell survival, differentiation, migration, and integration (Khazaei et al. 2014; Lu et al. 2014; Nutt et al. 2013; Pomeschchik et al. 2015; Romanyuk et al. 2015).

**PSC-Derived Oligodendrocyte Precursor Cells (OPCs)**

Oligodendrocyte death and chronic demyelination are features of the secondary injury cascade that follows the primary trauma after SCI (Kakulas 1999; Totoiu and Keirstead 2005). Adult OPCs have a limited capacity to regenerate or migrate, but a variety of stem cells, including endogenous adult NSCs, preferentially differentiate into oligodendrocytes that are capable of providing trophic support, remyelination and functional repair (Faulkner and Keirstead 2005; Gensert and Goldman 1997; Meletis et al. 2008). Functional recovery after NSC transplantation has been attributed in large part to OPC differentiation. ESC-derived OPCs have been transplanted after SCI with success, resulting in remyelination of host axons, improved electrophysiological and motor functions, and greater white and gray matter sparing (Brustle et al. 1999; Keirstead et al. 2005; Nistor et al. 2005; Sharp et al. 2010). Phase I/2 clinical trials for
the transplantation of human ESC-derived OPCs into SCI patients with thoracic and high cervical injuries are currently underway, with demonstrable neurological improvements observed in patients from the initial safety cohort (Khazaei et al. 2014).

**PSC-Derived Astrocytes**

Astrocyte diversity and the role of astrocyte phenotype on neural circuit formation and regeneration remains poorly understood (Clarke and Barres 2013; Zhang and Barres 2010). While reactive astrocytes actively inhibit regeneration through the glial scar, other endogenous astrocytes have pro-regenerative roles, including the formation of astrocyte bridges that precede axons extending through the inhibitory environment (Kawaja and Gage 1991; Nicaise et al. 2015; Taylor et al. 2006b). It is thus important to consider how the preparation of stem cell-derived astrocytes affects phenotype prior to transplantation; glial-restricted precursors that are pre-differentiated into astrocytes using different growth factor cocktails have opposite effects on recovery after transplantation (Davies et al. 2008). Astrocytes that differentiate from PSC-derived NSCs *in vivo* may contribute to or deter from the regenerative effects seen after transplantation depending on the method of NSC derivation, but the outcome is rarely linked to a specific astrocyte subtype. Protocols to obtain pro-regenerative astrocyte subtypes are still being developed, so studies that investigate the specific effect of PSC-derived astrocytes on spinal cord regeneration have not yet been published (Benveniste et al. 2005; Emdad et al. 2012; Falnikar et al. 2015; Roybon et al. 2013).

**PSC-Derived Motor Neurons**
MNs have some potential for cell replacement because their loss significantly impairs behavioral recovery in several traumatic and neurodegenerative conditions. Many protocols have been developed to obtain enriched, subtype-specific MN pools that closely resemble endogenous neurons and retain the ability to engraft in the developing spinal cord (Davis-Dusenbery et al. 2014). Efforts to transplant enriched MNs after SCI have been confined to embryonic or PSC-derived pMNs, since maturation comes with an intrinsic loss in plasticity needed to survive the hostile SCI environment. PSC-derived pMNs are able to survive, migrate, and differentiate after SCI transplantation in vivo, but the gliogenic nature of the injury site limits neuronal differentiation despite the ability of these pMNs to robustly differentiate into MNs in vitro (Erceg et al. 2010; Rossi et al. 2010). PSC-derived pMN transplantation also confers neuroprotective and neuroregenerative benefits akin to OPC transplantation; pMNs secrete neurotrophic factors that enhance endogenous axon sprouting and can result in functional improvements (Erceg et al. 2010; Rossi et al. 2010). Methods to direct maturation of progenitor populations or augment survival of mature neuronal transplants in vivo need improvement to take advantage of new PSC derivation methods.

**PSC-Derived Interneurons**

Local INs have been implicated in the formation of detour circuits that significantly contribute to functional recovery after partial SCI (Courtine et al. 2008; Flynn et al. 2011). Transplanted PSC-derived NSCs differentiate into a variety of INs that are capable of reconstructing functional neural relays, but, like astrocytes, the specific identity of those INs is rarely investigated beyond neurotransmitter expression profiles. Identifying the contributions of
unique subtypes towards regeneration is important, since they respond differentially after SCI (Flynn et al. 2011; Husch et al. 2012). The recent availability of protocols and transgenic ESC lines that enrich for specific IN populations should improve access to these subtypes for modeling and transplantation (Brown et al. 2014b; Iyer et al. 2016a; Kim et al. 2009; Murashov et al. 2005; Xu et al. 2015; Xu and Sakiyama-Elbert 2015b).

### 1.5 Genetic Strategies to Evaluate and Improve Stem Cell Transplantation

In 1979, Francis Crick, then a novice neuroscientist, wrote prescient remarks foretelling the impact that molecular biology would have on the study of the brain. He suggested that …a method that would make it possible to inject one neuron with a substance that would then clearly stain all the neurons connected to it, and no others, would be invaluable. So would a method by which all neurons of just one type could be inactivated, leaving the others more or less unaltered. (Crick 1979)

Genetic tools that allow us to visualize neuronal connectivity and to modulate activity in a single population have become realized in the 21st century, revolutionizing the way that we approach neuroscience research and therapy development. Here I touch on some key technologies, and offers some suggestions as to how they might be applied towards improving stem cell-derived therapeutics for SCI.
1.5.1 Targeted Genomic Engineering: Homologous Recombination to CRISPR/Cas9

The concept for targeted genomic engineering originated from studies in yeast and bacteria in the late 1970s investigating endogenous DNA repair mechanisms (Doudna and Charpentier 2014; Scherer and Davis 1979). Spontaneous double-stranded breaks in DNA are normally repaired by one of two pathways: homologous recombination, which enables precise DNA repair by using a homologous template sequence, or non-homologous end joining, wherein broken ends are ligated independently of a template, and thus can create irregular junctions. By the mid 1980s, several groups had shown that homologous recombination in mammalian cells could be manipulated to generate targeted insertions and mutations, ultimately leading to the generation of transgenic knock-out and lineage-traced mice created using engineered ESCs (Capecchi 2005; Folger et al. 1982; Mansour et al. 1990; Sauer and Henderson 1988; Smithies et al. 1985; Thomas and Capecchi 1987; Thomas et al. 1986; Yang et al. 1997). A major limitation to this approach is the extremely low probability of a naturally occurring double-stranded break at the desired gene site coupled with the high occurrence of non-homologous end joining compared to homologous recombination (about 1000-fold higher)(Capecchi 2005).

While the introduction of positive and negative selection markers improved screening methodologies to reduce workload (Mansour et al. 1990; Thomas and Capecchi 1987), strategies to create precise double-stranded breaks were sought as a means to improve genomic engineering efficiency (Doudna and Charpentier 2014). Technologies built on homing endonucleases first appeared in the mid 1990s with zinc finger nucleases (ZFNs), synthetic, modular DNA recognition proteins that when coupled with the restriction enzyme Fok1 could cleave genomic DNA specifically (Bibikova et al. 2003; Bibikova et al. 2002; Kim et al. 1996;
Miller et al. 1985; Pavletich and Pabo 1991). Though effective, the difficulty and expense in designing highly specific ZFNs prevented wide adoption. Similar problems plagued transcription activator-like effector nucleases (TALENs), which emerged as ZFN alternatives in the late 2000s with the discovery of transcription activator-like effectors (TALEs), amino acid sequences that can act as a DNA binding domain to a fused Fok1 enzyme (Boch et al. 2009; Christian et al. 2010; Moscou and Bogdanove 2009). While the mapping of TALEs for TALEN generation is comparatively straightforward, the assembly is labor intensive due to the repetitive sequence. Further optimization of TALEN technology was effectively halted by 2012 with the demonstration that the CRISPR-Cas9 system could efficiently target and edit the human genome with simple modifications to a guide RNA sequence.

CRISPRs (clustered regularly interspaced palindromic repeats) were first identified in E. coli in the late 1980s (Ishino et al. 1987), but a series of observations spanning from 2005-2011 uncovering the role of CRISPRs and cas (CRISPR-associated) gene encoded nucleases in adaptive defense against viral or phage infections and gene targeting were instrumental in the development of the CRISPR-Cas9 system (Bondy-Denomy and Davidson 2014; Doudna and Charpentier 2014; Gasiunas et al. 2012; Jinek et al. 2012; Makarova et al. 2011). In nature, CRISPRs encode guide RNAs that complex with Cas proteins, resulting in dramatic conformational changes that allow for recognition, binding, unwinding, and cleavage of double-stranded DNA. An efficient single guide RNA (sgRNA) system was first engineered in 2012, containing a Cas9 binding domain coupled with a short 20-nucleotide sequence that could be programmed to target any DNA sequence adjacent to a protospacer adjacent motif (PAM) (Gasiunas et al. 2012; Jinek et al. 2012). Subsequent studies in 2013 showed the extraordinary
efficacy of CRISPR-Cas9 mediated site-specific editing in human cells, ushering a tide of genomic engineering studies in a vast array of cell types and organisms. (Cong et al. 2013; Hsu et al. 2014; Jinek et al. 2013; Mali et al. 2013a; Mali et al. 2013b; Sander and Joung 2014). The impact of CRISPR-Cas9 technology in revolutionizing genetic engineering cannot be understated; in an astonishingly short span of time, CRISPR-Cas9 has evolved from an in vitro tool into a realistic gene therapy option to combat disease (Lin et al. 2014; Long et al. 2014; Wu et al. 2015; Yin et al. 2014). However, the potential for off-target effects remains a concern, as well as ethical issues surrounding the use of current CRISPR-Cas9 technology in human germline and somatic cells (Fu et al. 2013; Hsu et al. 2013; Lanphier et al. 2015; Liang et al. 2015; Pattanayak et al. 2013).

1.5.2 Imaging Neuronal Signaling: Genetically Encoded Calcium Indicators

Because of the ubiquity of calcium in intracellular signaling, and especially in neurons where calcium has essential roles in synaptic activity, the ability to visualize calcium transients is of enormous benefit (Grienberger and Konnerth 2012). One of the first calcium indicators, the bioluminescent photoprotein aequorin, was identified and isolated in the 1960s from the jellyfish Aequorea victoria (Ashley and Ridgway 1968; Shimomura et al. 1962). Synthetic absorbance dyes soon followed in the 1970s (Brown et al. 1975), but the development of more sensitive, hybrid chelator-fluorophores in the mid 1980s by Roger Tsien and others significantly widened the applicability of these dyes and buffers to neurological systems because of their relative ease of use and adaptability (Grienberger and Konnerth 2012; Tsien 1980). Protein-based genetically encoded calcium indicators (GECIs) have since become prominent in neuronal imaging, taking
the form of both FRET-based methods as well as engineered single fluorophore proteins rendered sensitive to physiological conditions. First developed in Tsien’s lab in the late 1990s, GECIs are attractive because of the potential to use them in vivo or ex vivo where traditional electrophysiological methods are ineffective for monitoring functional activity across specific neuronal populations (Grienberger and Konnerth 2012; Griesbeck 2004; Miyawaki et al. 1999; Miyawaki et al. 1997; Rose et al. 2014). The GCaMP family of single wavelength sensors in particular has been used by many groups to evaluate physiology in live animals because of its comparatively good signal-to-noise ratio, dynamic range, and response kinetics. They are engineered proteins based on circularly permuted GFP (cpGFP), calmodulin (CaM), and the calcium/CaM binding peptide M13; upon calcium binding, the CaM domain and M13 peptide interact to alter the environment of the cpGFP, thus shielding the chromophore from quenching activity (Akerboom et al. 2009). Since the development of the first GCaMP proteins in the early 2000s, multiple variants have been engineered to improve the speed and sensitivity of these indicators to better show action potential-associated signaling (Akerboom et al. 2012; Chen et al. 2013b; Ji et al. 2004; Nakai et al. 2001; Tian et al. 2009).

1.5.3 Manipulating Functional Activity: Chemogenetics and Optogenetics

Two significant technologies have allowed for the specific and reversible manipulation of neuronal firing both in vitro and in vivo: chemogenetics and optogenetics. While chemogenetic approaches seek to modulate neuronal activity by applying pharmacological agents to induce engineered receptor-ligand interactions, optogenetic tools rely on the ectopic expression of opsins proteins, which allow light-mediated control of neuronal firing. Their development has helped
target genetically and anatomically defined neuronal populations to investigate their role in awake, behaving animals, an enormous advance from traditionally invasive electrical stimulation methods.

The earliest examples of chemogenetics were based on manipulating G protein-coupled receptors (GPCRs), the largest class of molecules involved in neuronal signal-transduction. In a landmark study, Strader et. al. introduced site-directed mutations to the β2 adrenergic receptor, reducing the receptor’s affinity to its native ligand while enabling activation using a synthetic agonist (Small et al. 2001; Sternson and Roth 2014; Strader et al. 1991). RASSL (receptors activated solely by synthetic ligand) technology subsequently evolved, which focused on engineering receptors that could be used in vivo with highly potent, pre-existing synthetic drugs, including opioid receptor agonists which were used for the first engineered RASSL (Conklin et al. 2008; Coward et al. 1998; Sternson and Roth 2014). However, cyclic site-directed mutagenesis is labor intensive, and because many of the first RASSL ligands also activated endogenous receptors, there were inherent issues with experimentation due to constitutive activity in vivo. The next generation of chemogenetics, designated DREADDs (designer receptors exclusively activated by designer drugs), took advantage of evolutionary mutagenesis in yeast to engineer muscarinic receptors that were insensitive to their native ligand acetylcholine, but were potently activated by clozapine N-oxide (CNO), which has excellent pharmacokinetics in the CNS (Alexander et al. 2009; Armbruster et al. 2007; Bender et al. 1994; Rogan and Roth 2011; Schmidt et al. 2003). Bryan Roth’s group and others have been able to generate a family of DREADD-based muscarinic receptors activated by CNO, and which, when expressed in neurons, can induce different types of neuronal activity, including attenuation of
neuronal firing (hM4Di), depolarization and burst firing (hM4Dq), and augmentation of cAMP-mediated signaling (GsD) (Armbruster et al. 2007; Farrell et al. 2013; Guettier et al. 2009; Roth 2016; Sternson and Roth 2014; Urban and Roth 2015). New DREADDs that allow for chemogenetic activation of other signaling pathways being investigated; however, though DREADDs have the advantage of non-invasively directing neuronal function, the technology is generally disadvantaged by the lack of fine temporal control that is a hallmark of optogenetics. Chimeric, engineered ligand-gated ion channels (LGICs) offer better control due to their modularity and faster drug kinetics, but they are more suited to neuronal silencing in vivo than activation (Magnus et al. 2011; Sternson and Roth 2014). While DREADDs and LGICs represent the most established chemogenetic tools, others are under development, including engineered enzymes, kinases and dimerization technologies (Sternson and Roth 2014).

Because of the flexibility and timescale of control—both needed to manipulate spiking activity, which can occur over milliseconds—the use of optogenetics has been widely adopted. Though the study of microbial opsin-mediated channels began in the early 1970s (Oesterhelt and Stoeckenius 1971), the sub-field of optogenetics was sparked by a seminal report from Edward Boyden and Karl Deisseroth in 2005 which showed that by introducing channelrhodopsin-2, a light-sensitive cation channel, into neurons, the cells could be excited by photostimulation (Boyden 2011; Boyden et al. 2005). After absorbing light, the channel rapidly opens to allow monovalent and divalent cations to enter, thus creating a photocurrent that depolarizes the cell (Nagel et al. 2003). The Boyden and Deisseroth groups subsequently showed that expression of halorhodopsin or archaerhodopsin could be used to induce light-driven neuronal silencing, which occurs via hyperpolarization (Chow et al. 2010; Zhang et al. 2007). Many incremental changes
have since been made to improve the expression efficiency in different types of mammalian cells, as well to create novel, engineered opsins that respond to different frequencies of light and can thus be used in tandem with other genetically encoded fluorophores, including GECIs (Deisseroth 2015; Fenno et al. 2011; Zhang et al. 2011). The past few years have seen an acceleration in the number of groups using optogenetic tools to deconstruct neuronal function in the brain, spinal cord, and periphery, as well as working to engineer more sophisticated devices to deliver light effectively for therapeutic purposes (Ahmad et al. 2015; Boyden 2011; Bryson et al. 2016; Deisseroth 2015; Montgomery et al. 2015).

1.5.4 Engineering Stem Cells for Spinal Cord Injury

Approaches using genetic engineering in the context of spinal cord injury are numerous, and have been reviewed by others in some detail (Taha 2010; Walthers and Seidlits 2015). Of note is the broad use of viruses to transduce cells of interest, which I have not discussed in this introduction, but has become common in the neuroscience community to quickly and efficiently introduce a genetic manipulation both in vitro and in vivo (Betley and Sternson 2011; Davidson and Breakefield 2003). Here I will comment on only a few possible applications of targeted genetic engineering related to stem cells that work to either improve the therapeutic potential of stem cells or to help evaluate stem cell derived neuronal populations post-transplantation.

While biomaterials and viral vectors have oft been used to exogenously deliver pro-regenerative factors (Raspa et al. 2016; Thomas et al. 2015; Tuinstra et al. 2012; Tuinstra et al. 2014), cells can also be engineered as self-renewing drug delivery vehicles if appropriately modified. This presents an opportunity for sustained local delivery of growth factors or anti-
inhibitory molecules at the site of the injury without the need for synthetic particles (Blesch et al. 2002; Thuret et al. 2006; Walthers and Seidlits 2015). The inclusion of tetracycline-inducible systems can help control long-term gene expression and thereby mitigate issues associated with gene overexpression, including the “candy store effect” which can limit axon extension out of the graft (Blesch et al. 2002). Transducing stem cells in vitro prevents complications from direct in vivo gene manipulations, but can result in limited cell survival post-transplantation (Walthers and Seidlits 2015). Genetic engineering can also be used to direct differentiation of transplanted stem cells or endogenous adult NSCs and reactive astrocytes into more pro-regenerative glia or neurons (Corti et al. 2012; Guo et al. 2014; Tang and Low 2007). Transgenic modifications can enrich specific PSC-derived neuronal populations of interest and/or eliminate undifferentiated PSCs that could generate teratomas. Our lab has developed several transgenic ESC lines that use transcription factor-driven antibiotic resistance to obtain specific ventral spinal populations that are difficult to isolate from primary tissues, but may also suffer from low differentiation efficiencies (Iyer et al. 2016a; McCreedy et al. 2014a; McCreedy et al. 2012; Xu et al. 2015).

Optogenetic and chemogenetic techniques have been extensively used to determine behavioral roles for genetically defined neuronal populations (Deisseroth 2015; Kiehn 2016; Roth 2016); studies are now being conducted to investigate the potential use of these technologies to treat spinal cord injury (Ahmad et al. 2015). When combined with stem cells, they could be used to better evaluate functional connectivity at the host-graft interface, which has been historically been achieved using ex vivo electrophysiology preps or re-transection or excitotoxic ablation combined with open-field behavioral testing (Courtine et al. 2008; Bradbury et al. 2002; Lu et al. 2014). By expressing DREADDs or opsins in the stem cell transplant, these
types of assessments could be done noninvasively and reversibly, enabling long-term monitoring to determine the specific time course of rewiring, or the genetic identity of differentiated stem cells responsible for locomotor gains. Alongside physical rehabilitation, these techniques could also be used to actively promote rewiring, replacing gross electrical stimulation methods with more targeted stimulation (Creasey et al. 2004; Karadimas et al. 2016; Montgomery et al. 2016).

1.6 Concluding Remarks

Given their role in functional rewiring events following incomplete SCI, spinal INs represent an effective population to target for cell replacement therapies. This thesis concentrates on V2a INs, which have numerous roles in the lumbar CPG and respiratory circuits of the cervical spinal cord and hindbrain, and have an elevated, differential physiological response to SCI. However, difficulties identifying and obtaining INs from tissue has motivated the need for an alternative source for transplantation. Fortunately, the potential for stem cell-based therapies has never been greater. Ethical impediments caused by stem cell research have largely been mitigated by advancements in iPSC and direct reprogramming technologies. Human stem cells can be sourced from autologous patient tissue, permitting personalized strategies that reduce the risk of immune rejection as well as enabling disease modeling. In vitro methods to obtain specific spinal cell types have been made possible by the significant strides in research parsing spinal cord development and the genetic events that contribute to cell fate determination. After transplantation, stem cell-derived populations remyelinate host axons, administer trophic support, and contribute to relay circuits that promote functional recovery. New genetic
technologies have matured into powerful tools that can be used to engineer therapeutically valuable cell lines and to assess how stem cell-derived neuronal populations impact connectivity after transplantation.

This thesis details the development of a stem cell-based platform in order to study how V2a INs might play a role in regeneration after SCI. In Chapter 2, CRISPR/Cas9 technology is applied to generate a selectable ESC line to enrich for Chx10+ V2a INs following differentiation. Chapter 3 describes an in vitro aggregate culture system comparable to the ubiquitous dorsal root ganglion assay to evaluate whether pro-regenerative growth factors differentially affect ESC-derived V2a IN growth and functional maturation. Finally, in Chapter 4, different combinations of neuroaggregates are transplanted into a C3/C4 lateral contusion model of SCI to assess the viability of transplanting post-mitotic ESC-derived V2a INs and to determine whether they impact spontaneous respiratory recovery. The work herein contributes tools and models that significantly advance our ability to study V2a INs in the context of SCI.
Chapter 2

Generation of Highly Enriched V2a Interneurons from Mouse Embryonic Stem Cells

2.1 Abstract

Challenges in parsing specific contributions to spinal microcircuit architecture have limited our ability to model and manipulate those networks for improved functional regeneration after injury or disease. While spinal interneurons (INs) have been implicated in driving coordinated locomotor behaviors, they constitute only a small percentage of the spinal cord and are difficult to isolate from primary tissue. In this study, we employed a genetic strategy to obtain large quantities of highly enriched mouse embryonic stem cell (ESC)-derived V2a INs, an excitatory glutamatergic IN population that is defined by expression of the homeodomain protein Chx10 during development. Puromycin N-acetyltransferase expression was driven by the native gene regulatory elements of Chx10 in the transgenic ESC line, resulting in positive selection of V2a INs after induction and treatment with puromycin. Directly after selection, approximately 80% of cells are Chx10+, with 94% Lhx3+; after several weeks, cultures remain free of proliferative cell types and mature into normal glutamatergic neurons as assessed by molecular markers and electrophysiological methods. Functional synapses were observed between selected ESC-derived V2a INs and motor neurons when co-cultured, demonstrating the potential of these cells to form
neural networks. While ESC-derived neurons obtained in vitro are not identical to those that develop in the spinal cord, the transgenic ESCs here provide a unique tool to begin studying V2a INs in isolation for use in in vitro models of spinal microcircuits or for transplantation after spinal cord injury.

2.2 Introduction

Neural networks in the form of central pattern generators (CPGs) are capable of generating rhythmic motor outputs that are essential to a range of sophisticated locomotor behaviors, but the microcircuit architecture involved has been much harder to characterize due to the diversity of cell types and lack of spatial organization in the spinal cord (Arber 2012; Rybak et al. 2015). This is not only detrimental to our understanding of CPG circuitry but also hinders manipulations of those networks for improved functional outcomes after disease or trauma (Courtine et al. 2009; Harkema 2008a). Recent work using genetic ablation and ex vivo electrophysiological characterization of isolated spinal cord preparations has helped identify unique transcriptional markers to define the spinal interneuron (INs) populations that comprise these local spinal circuits (Arber 2012; Azim et al. 2014; Crone et al. 2008; Gosgnach et al. 2006; Jessell 2000; Kiehn 2006; Lanuza et al. 2004; Zhang et al. 2008). However, the dependence on animal models precludes high-throughput pharmacological testing or in vitro modeling of spinal circuitry which may aid in the development of targeted therapeutics that promote neural regeneration and plasticity. Here we describe a method to generate large quantities of highly enriched INs from embryonic stem cells (ESCs), focusing on the acquisition of V2a INs.
V2a INs are defined by expression of the homeodomain protein Chx10 and are involved in CPG and propriospinal networks in the spinal cord and respiratory centers of the hindbrain (Al-Mosawie et al. 2007; Azim et al. 2014; Crone et al. 2008; Crone et al. 2012; Dougherty and Kiehn 2010a; Dougherty and Kiehn 2010b; Lundfald et al. 2007; Peng et al. 2007). They are an ipsilaterally projecting glutamatergic premotor population with conserved locomotor functions in zebrafish and mice (Crone et al. 2008; Dougherty and Kiehn 2010b; Kimura et al. 2006). V2a INs are distributed homogeneously along the rostrocaudal axis of the spinal cord in early mouse embryos but are localized to the ventral horn in the adult (Dougherty and Kiehn 2010a; Francius et al. 2013). Genetic ablation studies have demonstrated their role in coordinating left-right alternation and skilled-reaching, as well as modulation of locomotor variability and rhythmic breathing (Azim et al. 2014; Crone et al. 2008; Crone et al. 2012; Crone et al. 2009; Dougherty and Kiehn 2010a; Zhong et al. 2010; Zhong et al. 2011).

Robust “highly enriched” neuronal cultures are desirable because they can provide mechanistic insights otherwise confounded by mixed culture conditions. V2a INs, among others, are difficult to isolate from primary tissue in part because they make up a relatively small fraction of the total cells in the spinal cord (Crone et al. 2008). Self-renewing pluripotent cells, such as ESCs, are an attractive alternative to sorting primary tissue because they can be differentiated into a variety of cell types in large quantities for in vitro study or transplantation. By adapting established motor neuron (MN) differentiation protocols (McCreedy et al. 2012; Wichterle et al. 2002), we have previously shown that directed differentiation of ESCs into V2a INs is possible by exposing embryoid bodies (EBs) to retinoic acid (RA); a weak sonic hedgehog (Shh) agonist, purmorphamine; and a Notch-inhibitor, DAPT (Brown et al. 2014a). However,
despite our ability to derive V2a INs from ESCs, post-mitotic Chx10+ cells constitute only ∼15% of the total cell population post-induction, which is further diluted as glial cells proliferate with time (Brown et al. 2014a). Methods including fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting have been used to isolate single cell populations, but they are limited by the availability of antibodies to lineage-specific surface antigens, which have not been identified for many ventral IN populations, and require dissociation processes that can be harmful to mature neurons. While FACS can be used with transgenic reporter or lineage tracing cells, it can significantly compromise the viability of mature neurons and retains the potential for contamination.

Transgenic selection of desired ESC-derived populations has proven to be an effective method to generate isolated populations of a variety of cell types, including progenitor motor neurons (pMNs) and MNs (Anderson et al. 2007; Li et al. 1998; Marchetti et al. 2002; McCreedy et al. 2014; McCreedy et al. 2012; Soria et al. 2000). Using lineage-specific promoters to drive antibiotic resistance, differentiation of the transgenic ESC line and subsequent antibiotic treatment results in highly enriched cultures that persist through maturation in vitro and in vivo after transplantation (McCreedy et al. 2014; McCreedy et al. 2012; McCreedy et al. 2014b). In this study, we generated a selectable “Chx10-Puro” ESC line and investigated whether it could be used to obtain V2a INs that were comparable to endogenous V2a IN populations.
2.3 Methods

2.3.1 ESC Culture

Transgenic and RW4 mouse ESCs were cultured on gelatin-coated T-25 flasks in complete media, consisting of Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies #11965-092, Carlsbad, CA) containing 10% newborn calf serum (Life Technologies #16010-159), 10% fetal bovine serum (Life Technologies #26140-079), and 1x Embryomax Nucleosides (Millipore #ES-008-D, San Francisco, CA). ESCs were passaged every two days at a 1:5 ratio in fresh complete media containing 1000 U/mL leukemia inhibitory factor (LIF; Millipore # ESG1106) and 100µM β-mercaptoethanol (BME; Life Technologies #21985-023).

Figure 2.1: Chx10-Puro cell line validation

(A) Schematic of Chx10-PAC targeting vector. (B) jPCR validation of PAC insertion into first exon of Chx10; the 2.4 kb fragment is an amplification from the endogenous 5’ UTR into the PAC cassette. (C) Copy number analysis of PAC in parental RW4 cells, Hb9-Puro ESCs (McCreedy *et al.* 2014), and Chx-10 Puro ESCs. GAPDH was used as a control.
2.3.2 Chx10-Puro Selection Vector

The targeting vector was constructed from a Gateway-compatible plasmid (pStart-K; Addgene #20346, Cambridge, MA) using a 750 bp SalI-Ascl fragment containing 5’ untranslated sequences and a 750 bp Ascl-Not1 fragment containing 3’ genomic sequences of Chx10 exon 1. A PAC/pGKneo dual resistance cassette was inserted between the two arms as previously described (McCreedy et al. 2012). The dual resistance cassette contains from 5’ to 3’: Ascl site, Kozak sequence, puromycin cassette with bgd polyA signal (PKO-Select Puro; Agilent Genomics, Santa Clara, CA), floxed phosphoglycerate kinase I promoter driving the neomycin phosphotransferase gene (PGK-neo) with bgd polyA signal, and Ascl site (Kozak 1986; Thomas and Capecchi 1987; Wu et al. 2008). Gateway recombination with the LR Clonase II Kit (Life Technologies #11791) was used to transfer aTTL-flanked regions into the pWS-TK3 vector, which contains the thymidine kinase gene for negative selection (Figure 2.1A) (Wu et al. 2008).

2.3.3 Electroporation and Clonal Analysis

A CRISPR/Cas9 system was used to insert our resistance cassette into the Chx10 locus with high efficiency. The vector containing the Chx10 guide RNA (gChx10) was generated by the Genome Engineering Core at Washington University in St. Louis. The Chx10 gRNA sequences target the ATG start site of Chx10 exon 1 and were placed into a derivative of Addgene plasmid #43860; no common SNPs were found and off target profiles were excellent with at least 3 bp of mismatch between the target and any other site in the genome, which dramatically reduces the probability of an off target cut (Veres et al. 2014). The p3s-Cas9HC vector contains the Cas9 open reading frame (Addgene plasmid #43945) (Cho et al. 2013).
1x10^7 RW4 ESCs were resuspended in electroporation buffer (20 mM HEPES pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM dextrose) with 8 µg Chx10-Puro selection vector, 1 µg Cas9 vector, and 1 µg gChx10 vector. Cells were electroporated at 0.23 kV and 960 µF in a 0.4 cm cuvette (Bio-Rad #165-2081, Hercules, CA) then plated on a 100 mm gelatin-coated petri dishes in complete media with LIF and BME for the cells to recover overnight. For the remainder of the expansion, cells were exposed to media containing LIF, BME, 150 nM fialuridine (FIAU, Moravek #M251, Brea, CA) and 40 µg/mL geneticin (G418, Life Technologies #10131) which was replaced every 2 days. After 10 days, single colonies were picked and plated into individual wells of a gelatin-coated 96-well plate. Clones were screened for correct insertion by junction PCR (Chx10Forward: GCCAACCGAGGAGAGCTAGAAGGTT; PACReverse: GCGCCAGGAGGCCTTCCATCTGT GCT) and expanded accordingly. The amplification region spanned from the endogenous 5’ UTR (outside of the Chx10-Puro selection vector homology arm) into the PAC cassette to confirm targeted insertion (Figure 2.2B). Fifty-six transgenic clones were screened for puromycin resistance in Chx10^+ interneurons following V2a induction (see V2a Induction and V2a Selection).

Transgenic clones with puromycin-resistant Chx10^+ INs were analyzed for PAC copy number by quantitative real-time polymerase chain reaction (qRT-PCR) using a customized TaqMan Copy Number Assay; mouse Tert was used as the endogenous control and the assay was prepared according to the manufacturer’s instructions. Calculations were completed using CopyCaller Software (v2.0, Applied Biosystems). The calculated copy number of each clone was
normalized to the calculated copy number for the Hb9-Puro ESC cell line, which was previously determined to contain a single PAC insertion (McCreedy et al. 2014).

2.3.4 Cre Excision

The PGK promoter and neomycin phosphotransferase genes were removed from an individual Chx10-Puro clone using the pTurboCre plasmid (gift from Timothy Ley). The pTurboCre plasmid (10ug) and an mRFP-expressing plasmid (1ug) were prepared for transfection using Lipofectamine 3000 (Life Technologies #L3000-001) according to the manufacturer’s instructions; cells were transfected for 24 hours. ESC cultures were then dissociated and plated sparsely (3 x 10^4 cells) in a 100 mm gelatin-coated petri dish with complete media, LIF, and BME. Single colonies were picked after 10 days and plated in 96-well gelatin coated plates. When confluent, the ESCs were split and tested for sensitivity to neomycin by exposure to 40 µg/mL geneticin for 5 days; those not sensitive were discarded. A single transgenic clone was used as the Chx10-Puro ESC line used for the remaining studies.

2.3.5 V2a Induction

V2a INs were obtained from ESCs using a “2/4+” induction protocol. EBs were formed by transferring 1 x 10^6 ESCs into 10 mL of DFK5 media on an agar-coated 100 mm petri dish for two days (‘). DFK5 is a DMEM/F12 base media containing 5% Knockout Serum Replacement (Life Technologies #10828-028), 50 µM Nonessential Amino Acids (Life Technologies #11140-050), 100 µM BME, 1:100 100x Insulin-Transferrin-Selenium (Life Technologies #41400-045), 100 µM BME, 1:200 100x EmbryoMax Nucleosides. EB media was then replaced with 10 mL DFK5 containing 10nM retinoic acid (RA; Sigma #R2625) and 1µM
purmorphamine (EMD Millipore #540223) for another two days (2\textsuperscript{+}). For the final 2 days (4\textsuperscript{+}), the media was replaced with DFK5 containing 10nM RA, 1\mu M purmorphamine and 5\mu M N-\{(N-(3,5-difluorophenacetyl-L-alanyl))-(S)-phenylglycine-t-butyl-ester (DAPT; Sigma #D5942). In induced V2a control cultures, the Shh antagonist cyclopamine (Cyc, 1 mM; Sigma #C4116) was used instead of purmorphamine.

2.3.6 V2a Selection and Culture

To determine cell viability after selection, transgenic ESCs induced using the 2\textsuperscript{/4}\textsuperscript{+} protocols were dissociated with 0.25\% Trypsin-EDTA (Life Technologies #25200-056) for 10 minutes with agitation and quenched with complete media containing 0.001\% DNAse (Sigma #DN25). Dissociated cells were counted and centrifuged at 1200 rpm for 5 minutes then re-suspended in a selection media of DFK5NB containing B-27 supplement (Life Technologies #17504-044), glutaMAX (Life Technologies #35050-061), 5 ng/mL glial-derived neurotrophic factor (GDNF; Peprotech #450-10, Rocky Hill, NJ), 5 ng/mL brain derived neurotrophic factor (BDNF; Peprotech #450-02, Rocky Hill, NJ), 5 ng/mL neurotrophin-3 (NT-3; Peprotech #450-03), and 2 \mu g/mL or 4 \mu g/mL puromycin in water (Sigma #P8833). DFK5NB is a combination of DFK5 media and Neurobasal Media (Life Technologies #21103-049) mixed at a 1:1 ratio. For selected and unselected cultures respectively, 5x10\textsuperscript{6} cells/cm\textsuperscript{2} and 5x10\textsuperscript{5} cells/cm\textsuperscript{2} were plated on individual laminin coated 24-well plates or 35mm dishes. After 24 hours of selection, the media was replaced with DFK5NB containing B27, Glutamax, NT-3, GDNF, and BDNF. The media was replenished as needed every two days.
2.3.7 Post-Selection Cell Viability

To determine the cell viability after 24 hours of selection with puromycin, cells induced using the 2-/4+ protocol were rinsed twice with DFK4 media to remove debris then incubated for 30 minutes in fresh DFK5 media containing calcein-AM (Life Technologies # C481). Fluorescent images were captured using a MICROfire camera attached to an Olympus IX70 inverted microscope. Quantification was completed by flow cytometry (n=4 biological replicates, see Flow Cytometry).

2.3.8 qRT-PCR

qRT-PCR was performed and analyzed as previously described (Brown et al. 2014a). Briefly, cDNA was synthesized using a High Capacity RNA-to cDNA Kit (Invitrogen). The cDNA was combined with TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA) and TaqMan Fast Advanced Master Mix (Applied Biosystems). qRT-PCR was performed using a Step One Plus Applied Biosystems thermocycler with the following protocol: 95°C for 20s; 40 cycles of 95°C for 1s and 60°C for 20s. There were n=3 technical replicates completed per run and n=4 biological replicates for each condition. For PAC expression, samples were treated with DNAse (Qiagen #7924, Valencia, CA) prior to analysis.

2.3.9 Immunocytochemistry

Immunocytochemistry staining was performed as previously described (McCreedy et al. 2014). Antibodies used include mouse anti-Chx10 (1:1000, Santa Cruz # sc-374151, Santa Cruz, CA), mouse anti-Lhx3 (1:1000, Lim3, Developmental Studies Hybridoma Bank (DSHB) #67.4E12, Iowa City IA), mouse anti-Hb9 (1:20, DSHB #81.5C10), mouse anti-Lim1/2 (1:50,
DSHB #4F2), mouse anti-Evx1 (1:50, DSHB #99.1-3A2), rabbit anti-beta-tubulin III (1:1000, βtubIII, Covance #811801, Princeton, NJ), rabbit anti-Ki67 (1:250, Abcam #ab15580), guinea pig anti-Vglut2 (1:2500, EMD Millipore #AB2251), mouse anti-MAP2 (1:250, EMD Millipore #AB5622), mouse anti-SV2 (1:100, DSHB #SV2), and secondary Alexa Fluor conjugated goat antibodies (1:200, Life Technologies). Nuclei were counterstained with Hoescht (1:1000, Life Technologies #H3569). For cell counting experiments, live cells were identified by phase contrast and Hoescht; only cells with apparent processes were included. Cell counts were performed using ImageJ (NIH); there were \( n=4 \) biological replicates for each condition with >450 cells counted per replicate.

2.3.10 Flow Cytometry

A modified 2/4+ protocol was used to obtain data on early expressing transcription factors in selected ESC-derived neuronal cultures. Briefly, EBs were dissociated at 2/3+ and 5\( \times \)10^6 cells were plated in individual wells of a laminin-coated 24-well plate in DFK5 media containing 10 nM RA, 1 µM purmorphamine, 5 µM DAPT, and 2 or 4 µg/mL puromycin for 24 hours. Surviving cells were dissociated with 0.25% Trypsin-EDTA for 5 minutes and quenched with complete media. Staining was conducted using the Transcription Buffer Set (Becton Dickinson #562725, Franklin Lakes, NJ) according to the manufacturer’s protocols with antibodies as described in “Immunocytochemistry.” Data was collected using a BD Canto II Flow Cytometer (Becton Dickinson). Between 10,000-100,000 events were recorded per condition (\( n \geq 4 \) biological replicates per condition) and analyzed using FloJo software (FlowJo, Ashland, OR); debris was removed from analysis using forward scatter versus side scatter and
Hoechst versus forward scatter plots. Gating parameters were set using control groups stained only with secondary antibodies.

### 2.3.11 Single-Cell Electrophysiology

Cells were induced using the 2/4 protocol, dissociated onto laminin coated 35 mm dishes at 2 \times 10^6 and selected for 24 hours with puromycin. Cultures were grown for up to 10 days post-selection in DFK5NB media with growth factors prior to recording. Cultures were perfused with Tyrode’s solution (150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, at pH 7.4). Whole-cell electrodes were filled with internal solution (140 mM K-glucuronate, 10mM NaCl, 5 mM MgCl₂, 0.2 mM EGTA, and 10 mM HEPES, at pH 7.4, supplemented with 5 mM Na-ATP and 1 mM Na-GTP). The open tip resistance was 2-5 MOhm. External solutions were delivered by local perfusion from an 8-barrelled pipette. Voltage-gated channel antagonists tetrodotoxin (TTX, 500 nM), tetraethylammonium chloride (TEA, 30 mM) and 4-aminopyridine (4-AP, 5 mM) were dissolved in Tyrode’s solution. Ligand-gated channel agonists were dissolved at 100 \mu M in 160 mM NaCl, 2 mM CaCl₂, 10 mM HEPES. Currents were recorded under voltage clamp with an Axopatch 200A amplifier, filtered at 1 kHz and digitized at 10 kHz using pClamp software (Molecular Devices, Sunnyvale, CA). Evoked synaptic currents were detected by simultaneous whole-cell recordings from adjacent pairs of cells in Chx10-Puro/Olig2-Puro mixed cultures. Brief steps to 0 mV from a holding potential of -80 mV elicited fast inward sodium currents that escaped voltage control in the axon of presynaptic cells and evoked excitatory postsynaptic currents in the adjacent cell, which was held at -80 mV.
2.3.12 V2a and pMN Co-Culture

Mixed Chx10-Puro and Olig2-Puro cultures were established to assess synapse formation between V2a INs and MNs. A constitutively active fluorescent reporter was knocked into the Rosa26 domain of the Chx10-Puro line as described above using the Ai9 plasmid (gifted by Hongkui Zeng, Addgene plasmid #22799)(Madisen et al. 2010), pTurboCre, the Cas9 plasmid, and Rosa26 guide RNAs (gR26) generated by the Genome Engineering Core at Washington University in St. Louis. Clones were screened for correct insertion by junction PCR (R26Forward: TCCCAAAGTCGCTCTGAGTT; CAGReverse: CCATCGCTGACAAAAATAAT) and expanded accordingly. The previously established Olig2-Puro ESC line contains the PAC gene under the expression of Olig2, which marks the pMN domain (McCreedy et al. 2012). The Olig2-Puro and Chx10-Puro ESCs were induced according to their respective 2-/4+ protocols (McCreedy et al. 2012), dissociated onto laminin (Life Technologies #23017-015) coated 35mm dishes at 2 x 10⁶ at a 1:1 ratio and selected for 24 hours in DFK5NB with 4 µg/mL puromycin. The media was replaced with DFK5NB containing B27, Glutamax, NT-3, GDNF, and BDNF and was replenished as needed every two days for up to 7 days. Neurobasal media containing the supplements and growth factors was used for the remaining duration of culture up to 4 weeks.

2.3.13 Statistical Analysis

Statistical analyses were performed using Statistica software (v5.5, StatSoft, Tulsa, OK). Significance was determined using Scheffe’s post hoc test for analysis of variance (ANOVA) with confidence as indicated. Average values reported with error bars are the standard deviation or standard error of the mean (SEM) as indicated.
Figure 2.2: Schematic showing PAC insertion into the Chx10 gene locus. (1) gChx10 guide RNAs mediate double stranded break at the ATG start codon. (2) Homologous recombination incorporated targeting vector cassette into the first exon. PAC expression was driven by the native Chx10 upstream promoters. The ubiquitously expressed PGK promoter drove neomycin phosphotransferase to screen cells with the incorporated dual cassette. (3) Cre recombinase was used to remove the PGK-neo cassette. (4) The final Chx10-Puro construct expresses PAC when Chx10 is expressed.

2.4 Results

2.4.1 Puromycin Resistance Coincides With Chx10 Expression In Transgenic ESC Line

In order to isolate V2a INs from a heterogeneous population of differentiated ESCs, we developed a genetic strategy to positively select for cells expressing the defining transcription factor Chx10 (Figure 2.2). CRISPR-assisted homologous recombination was used to target the first exon of the mouse Chx10 locus for puromycin N-acetyltransferase (PAC) insertion, thereby
placing antibiotic resistance under the control of the native Chx10 gene regulatory elements. A single clone with targeted genomic insertion (Figure 2.1B) and one copy of the PAC gene (Figure 2.1C) was treated with Cre recombinase to remove the neomycin selection cassette. The resulting Chx10-Puro ESC line was used for all subsequent analyses in this study.

To determine whether Chx10 expression resulted in the expression of PAC in the Chx10-Puro line, Chx10-Puro ESCs were differentiated using a 2i/4+ V2a induction protocol - cells were aggregated for two days to form EBs, then exposed to RA, purmorphamine, and DAPT for four days. Unmodified RW4 ESCs and cultures induced using the Shh antagonist Cyc were used as controls. qRT-PCR demonstrated that when induced with the 2i/4+ protocol, RW4 and Chx10-Puro cultures express comparable levels of Chx10 that are significantly higher than uninduced ESC and Cyc control cultures (p<0.0001) (Figure 2.3A). However, only induced Chx10-Puro cultures expressed PAC, which disappeared when Shh signaling was suppressed with Cyc. This suggests that PAC expression is activated with Chx10 expression in the Chx10-Puro line.
Figure 2.3: PAC expression in Chx10-Puro ESC line allows for enrichment of neuronal cells. (A) Chx10 and PAC mRNA expression in ESCs, ESCs induced with RA and puromycin (Pur), and ESCs induced with RA and cyclopamine (Cyc). (B) Schematic of 2/4 induction protocol followed by 24 hours of puromycin (Puro) selection. (C) Live cell viability assay (Scale bars = 100 µm) and (D) flow cytometry in ESCs, ESCs induced with RA and Pur, and ESCs induced with RA and Cyc following puromycin selection with 2 µg/mL or 4 µg/mL; control cultures without puromycin were run in parallel. *p<0.0001 compared to control group. (E) Phase contrast with tdTomato overlay demonstrating neuronal morphology in selected cultures (Scale bar = 50 µm).

To confirm cell viability post-selection, cultures were dissociated following the induction and plated on laminin-coated wells in DFK5NB media containing puromycin for 24 hours (Figure 2.3B). In the absence of puromycin, cells were viable in all conditions as evidenced by calcein AM staining (Figure 2.3C). Following puromycin selection, viable cells were only apparent in induced Chx10-Puro cultures (Figure 2.3G,H). The addition of puromycin killed all RW4 cultures as well as Chx10-Puro cultures induced with Cyc. Analysis by flow cytometry indicated 12.0 ± 3.6% and 11.0 ± 3.6% of cells were viable after selection with 2 µg/mL and 4 µg/mL puromycin, respectively, compared to 85.81 ± 3.79% viable cells in unselected cultures (p<0.0001) (Figure 2.3D). Surviving cells demonstrated neuronal morphology, including phase bright cell bodies and neurite extensions that are consistent with V2a induction (Figure 2.3E).

Table 2.1: Quantification of IN subtypes by flow cytometry. Flow cytometry analysis of ventral IN subpopulations in control and puromycin-selected cultures. Values presented as mean ± standard deviation. * denotes p<0.0001 compared to control group. # denotes p<0.05 compared to control group.

<table>
<thead>
<tr>
<th></th>
<th>Chx10</th>
<th>Lhx3</th>
<th>Hb9</th>
<th>Evx1</th>
<th>Lim1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>27.50 ± 9.21</td>
<td>53.93 ± 1.30</td>
<td>8.60 ± 4.52</td>
<td>23.80 ± 4.17</td>
<td>15.16 ± 1.53</td>
</tr>
<tr>
<td><strong>2 µg/mL</strong></td>
<td>80.37 ± 3.27*</td>
<td>93.96 ± 0.62*</td>
<td>1.17 ± 1.06#</td>
<td>0.61 ± 0.66*</td>
<td>3.21 ± 0.40*</td>
</tr>
<tr>
<td><strong>4 µg/mL</strong></td>
<td>70.25 ±12.90*</td>
<td>86.97 ± 5.83*</td>
<td>1.97 ± 1.42#</td>
<td>2.26 ± 1.16*</td>
<td>0.76 ± 0.64*</td>
</tr>
</tbody>
</table>
2.4.2 V2a IN Markers Are Enriched In Selected Chx10-Puro Cells

Immunocytochemistry and flow cytometry were used to confirm V2a IN identity in selected cultures using antibodies for the pan-neuronal marker (beta tubulin III (β-tubIII)) and defining transcription factors for V2a INs (Chx10, Lhx3), motor neurons (Hb9, Lhx3), V1 INs (Lim1/2), and V0 INs (Evx1, Lim1/2). The p2 progenitor marker Lhx3 is expressed in both V2 INs and immature MNs (Sharma et al. 1998), but expression of Lhx3 in post-mitotic cells has been used as a marker for V2a INs as it drives expression of Chx10 in certain contexts (Crone et al. 2008; Tanabe et al. 1998a; Thaler et al. 1999). To capture early, transient expression of these transcription factors, a shorter, modified 2/3+ induction protocol was used to ensure high protein expression post-selection for detection and quantification (Figure 2.4A).

Neuronal enrichment was quantified by counting the number of cell nuclei that co-localize with β-tubIII. Approximately 86.81 ± 5.40% of cells were β-tubIII+ in unselected control cultures; the percentage increased significantly to 98.27 ± 0.25% or 100% (p<0.05) following selection with 2 µg/mL or 4 µg/mL puromycin respectively. (Figure 2.4B). Analysis of the population distribution by flow cytometry confirmed that selection constitutes an enrichment of V2a cell identity (Figure 2.4C, Table 2.1, Figure 2.5). While unselected cultures contained cells from all the ventral IN subtypes (Figure C, Figure 2.6), a significant majority of cells were Chx10+ (Figure 2.4D) and/or Lhx3+ (Figure 2.4E) after treatment with puromycin (p<0.0001). The combination of staining and quantification suggest that there are negligible quantities of V0, V1 (p<0.0001) or MNs (p<0.05) that survive selection (Figure 2.4C, Figure 2.6).

While the intent of this study was to generate spinal V2a INs using Chx10 as the definitive transcription factor for identification, Chx10 is also expressed in the developing retina.
(Dhomen et al. 2006; Rowan and Cepko 2004). The expression of two retinal developmental markers, Rax and Six3 (Loosli et al. 1999; Muranishi et al. 2012), was assessed and they were not significantly upregulated in selected cultures compared to ESC controls, but Chx10 expression was significantly upregulated, as expected (Figure 2.7A-B). Cultures were stained for Isl1, which is a developmental marker for MNs in the spinal cord, but in combination with Chx10 and Lhx3 is also a marker for retinal bipolar cells (Elshatory et al. 2007). In selected cultures, 3.09 ± 0.93% and 0.47 ± 0.55% of cells were Isl1+ in cultures treated with 2 µg/mL and 4 µg/mL puromycin respectively, which were significantly lower ($p<0.05$) compared to 8.10 ± 1.78% of cells in unselected induced cultures (Figure 2.7C-D). Interestingly, using a higher concentration of puromycin resulted in a lower percentage of cells expressing V2a markers (Figure 2.4C), but also significantly reduces the number cells that express Isl1 compared to using 2 µg/mL puromycin ($p<0.05$). These more highly enriched (4 µg/mL puromycin) cultures were used for all maturation and electrophysiology experiments.
Figure 2.4: Characterization of Chx10-Puro ESC-derived subpopulations. (A) Schematic of modified 2-/4+ induction protocol. (B) Counting analysis of β-tubulin III (β-tubIII) distribution in control and puromycin-selected cultures. *p<0.05 compared to control group. (C) Flow cytometry analysis of ventral IN subpopulations in control and puromycin-selected cultures. *p<0.0001 compared to control group. #p<0.05 compared to control group. (D) Chx10 and (E) immunofluorescence analysis in control and puromycin-selected cultures. Scale bars = 200 µm.
Figure 2.5: Flow cytometry gating analysis. Representative flow cytometry analysis of IN subtypes and proliferation marker Ki67 in unselected (gray) and selected (black) differentiated Chx1-Puro cultures.
Figure 2.6: Immunocytochemistry analysis of Chx10-Puro ESC-derived ventral IN subpopulations. (A) Hb9, (B) Evx1, and (C) Lim1/2 immunofluorescence analysis in control and puromycin-selected cultures. Scale bars = 200 µm
Figure 2.7: Characterization of retinal markers in selected Chx10-Puro cells. (A) Chx10, (B) Rax and Six3 mRNA expression in unselected and selected Chx10-Puro ESCs after modified 2-/3+ protocol (C) Quantification of Isl1 in control and puromycin-selected cultures. *p<0.05 compared to control group, **p<0.05 compared to control group and 2 µg/mL puromycin selection. (D) Immunofluorescence analysis in control and puromycin-selected cultures for Isl1. Scale bars = 200 µm
2.4.3 Selected Chx10-Puro Neurons Are Post-Mitotic And Achieve Functional Maturity

Because neuronal differentiation of ESCs can give rise to proliferative cell types that reduce the purity of the culture, selected Chx10-Puro cultures were evaluated for mitotic activity. An antibody for the proliferation marker Ki67 was used to detect and quantify mitotic cells via immunocytochemistry and flow cytometry (Figure 2.8A,B Figure 2.5). Ki67$^+$ cells comprised 15.13 ± 2.58% of cells observed in unselected cultures, which was significantly decreased when treated with 2 ug/mL (1.80 ± 0.10%) or 4ug/mL (0.83 ± 0.29%) puromycin ($p<0.0001$). Unselected cultures also visibly showed a rapid increase in the number of cell nuclei over 2 weeks (Figure 2.8D).

Most V2a INs in vivo express excitatory vesicular glutamate transporter 2 (Vglut2) (Lundfald et al. 2007). To confirm functional maturation of selected Chx10-Puro cultures, cells were induced using the 2$^+/4^+$ protocol, dissociated onto laminin-coated plates and selected for 24 hours with puromycin, then grown for 14 days post-selection in DFK5NB media with growth factors (Figure 2.8C). Selected cells abundantly express Vglut2 and the mature dendritic marker, microtubule associated protein 2 (MAP2) (Figure 2.8D). Vglut2$^+$ neurons appear to be the majority of cells in selected cultures compared to only a small fraction in unselected cultures. Taken together, these data suggest that we have generated highly enriched, post-mitotic V2a INs.
Figure 2.8: Maturation of post-mitotic V2a INs. (A) Immunofluorescence analysis of the proliferation marker Ki67 in unselected and selected cultures directly after selection. (B) Flow cytometry analysis of Ki67 in control and puromycin-selected cultures. *p<0.0001 compared to control group. (C) Schematic of 2-/4+ induction protocol, selection, and extended-culture. Media was replaced with DFK5NB with growth factors directly after selection for up to two weeks post-selection. (D) Mature Vglut2, NF, and MAP2 staining in unselected and selected cultures after two weeks in culture.
2.4.4 Selected V2a INs Demonstrate Mature Electrophysiological Profiles

Whole cell current and voltage clamp recordings were performed to evaluate the functional maturity of cells in puromycin-selected cultures up to 12 days post-selection (d12). Cell capacitance, which is proportional to surface area, was low on d2 (17.1±1.95 pF, n=5) but increased to approximately 30 pF by d3 and remained relatively constant through d12 (Table 2.2). Input resistance was highest on d2 (2.22±0.66 GΩ, n=5) and declined as cells matured (415±80 MΩ, d12, n=7). Cell resting potentials were relatively depolarized at early time points after selection, but by d9-d12 the mean V_rest was approximately -50 mV. When membrane potential was maintained near -60 mV by steady current injection under current clamp nearly all cells with neuronal morphology were capable of firing action potentials upon stimulation with a depolarizing current pulse (Figure 2.9A). Of these, 35.7% fired single action potentials, 35.7% fired several action potentials near the beginning of an 800 msec pulse but then adapted, while the remaining 28.6% exhibited tonic firing throughout the depolarizing pulse. Under whole-cell voltage clamp, depolarizing steps from -80 mV elicited fast inward currents that were sensitive to blocking by the voltage-gated sodium channel antagonist tetrodotoxin (TTX, 0.5 µM). Traces in Figure 2.9B show TTX-sensitive current obtained by subtracting current recorded in TTX at each test potential from current recorded in control solution. In addition, all cells displayed transient and sustained outward currents mediated by voltage-gated potassium channels (Figure 2.9C, D). Consistent with previous work in other cell types (Bean 2007), the organic cation tetraethylammonium (TEA) blocked sustained currents, whereas transient currents were selectively inactivated at a holding potential (V_h) of -40 mV (Figure 2.9C) or by application of 4-aminopyridine (data not shown). The transient outward currents required less depolarization for
activation as shown in the plots of peak and steady-state (SS) outward currents as a function of test potential (Figure 2.9D).

To test for functional neurotransmitter receptors, cells were exposed to 100 µM selective agonists for AMPA/kainite, NMDA, glycine and GABA. Agonist activated inward currents were observed while holding cells at a fixed potential of -80 mV (Figure 2.10A). As the cells matured from d3 to d20 or more, the amplitude of these agonist-gated currents increased (Figure 2.10B). Agonist-evoked currents were also recorded as the membrane potential was slowly ramped between -110 and +110 mV (Figure 2.10C). Currents elicited by the inhibitory transmitters GABA and glycine reversed polarity near the estimated equilibrium potential for chloride ions (-54 mV) given the composition of our internal and external solutions. In contrast, the currents evoked by NMDA and kainate reversed much closer to 0 mV, consistent with monovalent cation permeability.
Figure 2.9: Action potentials and voltage-gated currents in selected Chx10-Puro cells. (A) Action potential firing patterns recorded on d9 in 3 different selected cells stimulated with 800 msec square pulse current injections. (B) Currents mediated by voltage-gated sodium channels sensitive to tetrodotoxin (TTX) on d2 evoked by steps from a holding potential of -80 mV to test potentials ranging from -60 to +25 mV. Traces show the difference between currents recorded in the absence and presence of 0.5 µM TTX. Peak inward current plotted as a function of test potential. (C) Transient and sustained outward potassium currents on d4 evoked by 130 msec voltage steps from holding potentials of -80 mV (above) or -40 mV (below) to test potentials ranging from -100 mV to +60 mV in the presence of 0.5 µM TTX. Exposure to 30 mM tetraethylammonium (TEA) blocked the sustained current, whereas holding at -40mV inactivated the transient current. (D) Current-voltage relations for transient (peak TEA) and sustained (SS) outward currents.
Figure 2.10: Currents activated by transmitter receptor agonists in selected Chx10-Puro cells. (A) Whole-cell currents evoked by brief application of 100 µM kainate, GABA, glycine or NMDA (plus 1 µM glycine) as indicated by the filled bars. Holding potential, -80 mV; 9 days after puromycin selection (* denotes significant difference from d3 and d9, # denotes significant difference from d3). (B) Peak agonist-gated current at -80 mV (mean ± SEM) recorded in cells 3, 9 or more than 20 days after puromycin selection. (C) Agonist-evoked currents recorded during voltage ramps from -110 to +110 mV at 1.2 mV/msec, 28 days after puromycin selection. GABA and glycine evoked currents that reversed polarity at -53.8 ± 1.7 mV (n=4) and -55.3 ± 1.7 mV (n=4), respectively, consistent with activation of chloride-selective channels. Currents evoked by kainate and NMDA reversed polarity at 5.9 ± 2.6 mV (n=4) and 19.8 ± 4.1 mV (n=4), respectively, consistent with monovalent cation permeability.
Table 2.2. Electrophysiological properties of selected Chx10-Puro cells. Values presented as mean ± SEM (number of cells) (* denotes significantly different from d2-4 by t-test, Mann-Whitney rank sum test, or Z-test). Cell capacitance and input resistance were determined from 10 mV voltage clamp steps from a holding potential of -80 mV. First spike latency, amplitude, absolute amplitude, threshold and half-width were determined for the first spike recorded at threshold depolarization. In addition, 800 msec depolarizations that elicited spiking with an average frequency of 10 Hz were used to measure 1st latency, instantaneous frequency from the first inter-spike interval (ISI), and frequency adaptation (ratio of last to first ISI). Sag in voltage responses was determined for 800 msec hyperpolarizing current injections from -60 mV.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>d2-4</th>
<th>d9-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacitance (pF)</td>
<td>33.1 ± 3.2 (19)</td>
<td>29.8 ± 2.1 (38)</td>
</tr>
<tr>
<td>Input Resistance (GOhm)</td>
<td>1.7 ± 0.24 (19)</td>
<td>0.57 ± 0.12 (38) *</td>
</tr>
<tr>
<td>Tau (msec)</td>
<td>52.1 ± 7.1 (19)</td>
<td>22.8 ± 9.1 (38) *</td>
</tr>
<tr>
<td>V rest (mV)</td>
<td>-37.6 ± 1.5 (18)</td>
<td>-51.2 ± 1.8 (28) *</td>
</tr>
<tr>
<td>% V rest &lt; -50 mV</td>
<td>5.60%</td>
<td>53.5% *</td>
</tr>
<tr>
<td>Rheobase (pA)</td>
<td>13.1 ± 6.3 (7)</td>
<td>49.7 ± 12.0 (21) *</td>
</tr>
<tr>
<td>1st spike latency (msec)</td>
<td>91.1 ± 22.4 (7)</td>
<td>76.1 ± 15.0 (21)</td>
</tr>
<tr>
<td>1st spike amplitude (mV)</td>
<td>70.4 ± 7.1 (7)</td>
<td>92.5 ± 4.1 (21) *</td>
</tr>
<tr>
<td>1st spike overshoot (mV)</td>
<td>11.9 ± 6.8 (7)</td>
<td>31.4 ± 4.1 (21) *</td>
</tr>
<tr>
<td>1st spike threshold (mV)</td>
<td>-38.9 ± 1.9 (7)</td>
<td>-37.4 ± 1.4 (21)</td>
</tr>
<tr>
<td>1st spike width (msec)</td>
<td>6.5 ± 0.9 (7)</td>
<td>2.7 ± 0.3 (21) *</td>
</tr>
<tr>
<td>10 Hz 1st latency (msec)</td>
<td>41.9 ± 5.9 (4)</td>
<td>31.4 ± 3.1 (14)</td>
</tr>
<tr>
<td>10 Hz 1st frequency (Hz)</td>
<td>11.3 ± 0.5 (4)</td>
<td>13.9 ± 1.3 (14)</td>
</tr>
<tr>
<td>10 Hz frequency adaptation</td>
<td>1.3 ± 0.03 (4)</td>
<td>1.4 ± 0.14 (14)</td>
</tr>
<tr>
<td>10 Hz after potential (mv)</td>
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<td>-0.15 ± 0.62 (14)</td>
</tr>
<tr>
<td>Sag at -90 mV (mV)</td>
<td>2.4 ± 1.0 (4)</td>
<td>2.4 ± 0.72 (14)</td>
</tr>
<tr>
<td>Peak I Na (nA)</td>
<td>-1.87 ± 0.27 (16)</td>
<td>-2.29 ± 0.22 (29)</td>
</tr>
<tr>
<td>Vm for peak I Na (mV)</td>
<td>-26.6 ± 2.0 (16)</td>
<td>-32.6 ± 1.6 (29) *</td>
</tr>
</tbody>
</table>
Overall, the membrane properties of our selected Chx10-Puro cells are broadly consistent with previous electrophysiological characterization of V2a interneurons in acute tissue slice and isolated spinal cord preparations (Dougherty and Kiehn 2010a; Zhong et al. 2010). Recordings from native neurons in these preparations have revealed a variety of action potential patterns including tonic, adapting and single spike firing, as are also seen in our cultures.

2.4.5 Synapses Observed in Mixed V2a and pMN Culture

The ability of ESC-derived neuronal populations to form synapses is critical for their use in cell therapy or for in vitro modeling of neural circuitry. Endogenous V2a INs primarily synapse onto MNs, but have also been observed to synapse onto other V2a INs. To model these interactions, selected ESC-derived V2a INs and pMNs were co-cultured for up to 4 weeks and examined for functional connectivity (Figure 2.11A-D). A previously established Olig2-Puro ESC line was used to obtain a highly enriched pMN culture, which gives rise to a variety of cell types including MNs, oligodendrocytes, and astrocytes. The presence of glia has been shown to be beneficial to spontaneous electrical activity and for improved synapse formation in culture (Boehler et al. 2007; Pfrieger and Barres 1997). A constitutively-active red fluorescent reporter was knocked into the Chx10-Puro line to identify V2a INs in co-culture conditions (Figure 2.11E-G); MNs were identified by their distinct morphology (Figure 2.11B). Robust staining for synaptic vesicles (SV2) was observed in both V2a INs and MNs after 3 weeks; 98.67 ± 1.03% of Vglut2⁺ cells in these cultures co-stained with SV2 (Figure 2.11H-J).
Figure 2.11: Chx10-Puro and Olig2-Puro co-culture. (A) Schematic of 2-/4+ induction protocol for Chx10-Puro and Olig2-Puro cells, followed by selection and up to four weeks of co-culture. Media is switched from DFK5NB to Neurobasal after one week. (B) Phase contrast and (C,D) Hoescht staining of mixed culture demonstrates variety of cell morphologies and cell debris. (E) TdTomato allows for identification of Chx10-Puro cells. (F,G, H) Vglut2 staining appears almost exclusively in V2a INs. (I,J) SV2 staining is prevalent in most neurons and indicative of synaptic activity. Scale bars = 200 µm.
To test for functional synaptic connections, we performed simultaneous whole-cell recordings from adjacent pairs of cells on d21 to d31 (Finley et al. 1996). Both cells were held at -80 mV and brief (5-10 msec) voltage steps to 0 mV were delivered at 0.2 Hz, first to one cell and then the other. As shown in Figure 2.12, the voltage steps in a labeled Chx10-Puro cell elicited inward sodium currents that escaped from voltage control in the presynaptic axon and evoked excitatory postsynaptic currents (EPSCs) in the adjacent Chx10-Puro cell. In all, we obtained successful simultaneous recordings from 9 cell pairs with 18 potential presynaptic cells, including 13 labeled Chx10-Puro cells and 5 unlabeled cells. Upon stimulation, most of the cells evoked EPSCs (11 of 13 labeled Chx10-Puro cells and 3 of 5 unlabeled cells) with an average amplitude of 520 ± 170 pA and a mean synaptic delay of 2.0 ± 0.2 msec from the peak of presynaptic inward current to the initial rise of the EPSC. The remaining 2 labeled Chx10-Puro cells and 2 unlabeled Olig2-Puro cells failed to evoke any postsynaptic response, suggesting either they did not make functional contact with the adjacent cell or they did not release a transmitter capable of eliciting fast postsynaptic currents. Importantly, the AMPA/kainate receptor antagonist NBQX (30 µM) blocked synaptic transmission mediated by all of the presynaptic Chx10-Puro cells, as well as the 3 unlabeled presynaptic cells, confirming that they exhibit an excitatory glutamatergic phenotype. Superfusion with the GABA_A receptor antagonist bicuculline methiodide (200 µM) did not affect evoked synaptic transmission in any of our paired recordings, but did block spontaneous IPSCs that were observed in several of the recorded cells in mature Chx10-Puro/Olig2-Puro mixed cultures. Similarly, NBQX also blocked spontaneous EPSCs observed while recording from individual cells in mixed cultures.
Figure 2.12: Evoked excitatory synaptic transmission between selected Chx10-Puro cells. Simultaneous whole-cell recordings of pre-and postsynaptic currents from two adjacent Chx10-Puro cells 31 days after puromycin selection. Voltage steps to 0 mV from a holding potential of -80 mV elicited inward sodium current, and uncompensated capacity transients, in the presynaptic cell. Excitatory synaptic currents (EPSCs) were evoked in the postsynaptic cell with a delay of 3.2 msec, measured from the peak of presynaptic sodium current to the initial rise of the EPSC. Exposure to the AMPA/kainate receptor antagonist NBQX (30 µM) completely blocked the EPSC, while the GABA_A receptor antagonist bicuculline methiodide (0.2 mM) had no effect. Each panel shows 3 superimposed traces in each condition. Presynaptic stimuli were delivered at 0.2 Hz; postsynaptic holding potential, -80 mV.

2.5 Discussion

2.5.1 Chx10-Puro Mouse ESC Line Yields Highly Enriched V2a INs

While optimized induction protocols have been developed to obtain several ventral spinal populations from ESCs (Brown et al. 2014a; McCreedy et al. 2014; McCreedy et al. 2012; Wichterle et al. 2002; Xu and Sakiyama-Elbert 2015a), the heterogeneity of resulting cultures make the study or transplantation of single cell types unfeasible. By using defining transcription factor promoters to drive puromycin resistance in ESC-derived cell populations, our lab and
others have been able to positively select for enriched populations of beta cells, cardiomyocytes, endothelial cells, neurepithelial progenitors, pMNs, and MNs (Anderson et al. 2007; Li et al. 1998; Marchetti et al. 2002; McCreedy et al. 2014; McCreedy et al. 2012; Soria et al. 2000).

Here we generated a Chx10-Puro ESC line and confirmed that, after induction and puromycin treatment, the vast majority of viable cells were Chx10⁺ and Lhx3⁺. By modulating the concentration of puromycin, contamination due to other ventral cell types and Chx10⁺/Lhx3⁺/Isl1⁺ retinal bipolar cells could be reduced. Within two weeks, selected Chx10-Puro cells mature into Vglut2⁺ glutamatergic neurons; Vglut2 presents as punctate staining along the neurite surface, however strong cytosolic staining was also observed at early time points. This may be caused by an upregulation of protein expression during early stages of maturation, or, in selected cultures, due to a lack of supportive cells that aid in maturation. Within four weeks of culture (Figure 2.11E-G), the cytosolic component is reduced. We also report a higher yield of Chx10⁺ cells in unselected cultures than previously published (Brown et al. 2014a), likely because the cells are maintained as EBs for the duration of the induction.

Our data suggests 80-90% purity in selected Chx10-Puro cultures, but it is probable that quantification by flow cytometry is an underestimation of true population counts and the purity of the selected cultures is likely to be higher than we observed due to transcription factor inactivation. While increasing the concentration of puromycin was expected to improve the yield of cells positive for V2a markers, instead we saw a downward trend despite the elimination of potential retinal cells marked by Isl1 expression and other ventral markers. A rapid decrease in the number of Chx10 and Lhx3 expressing cells was also observed with time (data not shown). Given that selected cultures are not overtaken by proliferative cell types and remain primarily
glutamatergic neuronal cells when mature, it is conceivable that when selected with 4 µg/mL puromycin, early V2a INs and retinal cells die before PAC expression is sufficient to counter puromycin activity, and that the surviving cells analyzed begin to lose V2a marker expression but represent a more mature V2a population. This is at odds with endogenous cells in animal models, where immunohistochemistry has been used to identify Chx10⁺ and Lhx3⁺ cells in spinal cord slices into maturity (Al-Mosawie et al. 2007; Crone et al. 2008; Crone et al. 2009; Dougherty and Kiehn 2010a; Dougherty and Kiehn 2010b; Lundfald et al. 2007). Lineage tracing using cre-recombinase would enable a clearer delineation of total subtype yields in ESC-derived cultures. Selected Chx10-Puro V2a INs also exhibited morphological and electrophysiological heterogeneity consistent with observations ex vivo, which seems to imply at least some degree of diversity (Al-Mosawie et al. 2007; Dougherty and Kiehn 2010a; Dougherty and Kiehn 2010b; Kimura et al. 2006). Several dozen transcription factors have been detected to identify discrete ventral IN subpopulations in the last few years alone (Francius et al. 2013), including at least one functionally distinct subpopulation of Chx10⁺ cells, a Shox2+/Chx10⁺ population, termed V2d (Dougherty et al. 2013). The availability of markers to classify V2 IN subpopulations is of major interest and would help to further characterize the purified population.

2.5.2 Functional Activity In Purified ESC-Derived V2a Populations Mimics Ex Vivo Data

Most native V2a interneurons in acute spinal cord preparations appear capable of spiking repeatedly throughout the duration of a prolonged depolarizing pulse, although a substantial minority exhibit other firing patterns including burst firing that adapts and single spiking (Dougherty and Kiehn 2010a; Zhong et al. 2010). Our selected Chx10-Puro cells also exhibit a
range of action potential firing, as well as other electrophysiological properties that are generally consistent with work on native V2a cells. Maturing selected Chx10-Puro cells expressed receptors for the major fast excitatory and inhibitory spinal neurotransmitters, suggesting that they should be capable of functional integration into spinal circuits.

V2a INs *in vivo* play a critical role in CPGs, networking with other cell types in order to achieve coordinated locomotion (Azim *et al.* 2014; Crone *et al.* 2008; Crone *et al.* 2012; Crone *et al.* 2009; Zhong *et al.* 2010; Zhong *et al.* 2011). In the present study, we demonstrate the ability of selected Chx10-Puro V2a INs to make functional excitatory synapses onto each other, as well as onto presumptive MNs. In the instances where there was no apparent connection between Olig2-Puro cells and adjacent labeled Chx10-Puro cells, it is possible that the unlabeled Olig2-Puro cells were MNs releasing acetylcholine, which did not acutely activate channels and thus did not produce a fast post synaptic current. The connections observed during paired recordings were likely monosynaptic because of their large amplitudes and short synaptic delays. In all cases tested, the AMPA/kainate receptor antagonist, NBQX, produced complete block of evoked transmission, demonstrating that the transmitter released by presynaptic Chx10-Puro cells activates postsynaptic glutamate receptors. Thus, our paired recordings provide conclusive evidence that selected Chx10-Puro cells are glutamatergic.

Previous work by Zhong *et al.* (2010) provided evidence for selective electrical coupling between V2a INs with similar action potential firing characteristics, a feature that might help to coordinate rhythmic activity. We saw no examples of electrical coupling in our paired recordings either from 9 cell pairs in mature Chx10-Puro/Olig2-Puro co-cultures (d20 to d31), or in preliminary recordings from 6 pairs in d10 Chx10-Puro cultures; however, we were not able to
characterize the spiking properties of these cells to determine whether or not both cells in a pair displayed similar firing patterns.

2.5.3 Chx10-Puro Cells For In Vitro Modeling

While most in vitro studies that use neuronal cell cultures have investigated functional properties, advances in culture methods, geometric and spatial patterning, instrumentation, and signal processing make possible “lab on a chip” technologies for brain and spinal cord (Wheeler and Brewer 2010). V2a INs occupy a significant role in the CPG and, compared to other IN types, have well characterized network properties that make them an attractive population with which to design an experimental platform (Dougherty and Kiehn 2010a). A minimalist approach could be taken, whereby V2a INs are observed in isolation, but transcription-factor driven selectable ESC lines could also be used modularly to generate complex cultures that remain well defined. We demonstrate the feasibility of such an approach here using both the Chx10-Puro and Olig2-Puro ESC lines to create a simple model of V2a-MN interactions. Because ESC induction protocols can be manipulated to enrich from distinct anatomical regions (Lippmann et al. 2015; Okada et al. 2004), there is a significant degree of flexibility possible using selectable lines.

A general caveat in the use of pluripotent stem cells for modeling or therapy is the gap between neurons obtained in the dish and those that develop normally in vivo. As evidenced by the selected Chx10-Puro cultures generated in this study, there remain some phenotypic and functional differences between ESC-derived V2a INs and endogenous cells. Some variation is expected, especially given the abnormal isolation of these neurons during a critical period of maturation, and it is possible that a longer duration of maturation or optimization of culture conditions might improve comparability. However, it is similarly possible that the significant
differences in development cannot be overcome and the cells here merely mimic V2a IN properties while incapable of the complex functional activity expected of endogenous V2a INs. Compounding the issue of characterization is a lack of comprehensive genetic profiles to identify functionally distinct IN subtypes. Creating tools to investigate these potential differences is beneficial to our understanding of spinal cord development and for improving stem cell therapies.

2.6 Conclusions

By knocking PAC into the Chx10 locus of mouse ESCs, the addition of puromycin to differentiated ESC cultures killed Chx10 negative cells. V2a IN enrichment was evident directly after selection of differentiated “Chx10-Puro” cells and persisted through maturation into functional glutamatergic neurons. Electrophysiology demonstrated that selected V2a INs are capable of spontaneously firing action potentials and respond to a range of agonists. Finally, our selected V2a INs form synapses with each other and with MNs in co-cultures. Together our findings suggest that selected Chx10-Puro V2a INs, while not identical to native V2a INs, are broadly comparable and can be a powerful resource for in vitro modeling of neural networks, investigation of neuronal development and diversification, and targeted cell therapies.
Chapter 3

An *In Vitro* Aggregate Culture System Investigating the Response of V2a Interneurons to Neurotrophins and Co-Culture with Progenitor Motor Neurons

3.1 Abstract

Combining growth factor treatments with cell therapy can dramatically improve functional recovery following spinal cord injury (SCI). However, efforts to optimize these treatments to target plasticity in locomotor-relevant populations has been modest, in part because of the reliance on animal models to evaluate regeneration in the central nervous system (CNS). Here we describe an *in vitro* culture platform akin to the ubiquitous dorsal root ganglion (DRG) assay for embryonic stem cell (ESC)-derived spinal populations. Enriched ESC-derived V2a interneurons (ESC-V2a INs) and ESC-derived progenitor motor neurons (ESC-pMNs) were cultured as spherical aggregates to generate a cellular microenvironment more closely related to that *in vivo* compared to 2D monolayer culture. We were able to apply common methodologies including the neurite extension assay, qRT-PCR and calcium imaging to investigate the effects of growth factor treatments on these isolated spinal populations. We used this platform to test whether V2a IN axon growth could be stimulated in a dose-dependent manner by glial-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3),
and platelet-derived neurotrophic factor (PDGF) and/or by co-culture with pMNs. ESC-V2as did not demonstrate any appreciable dose dependent responses to the growth factors applied, but co-culture with ESC-pMNs improved neurite extension, long-term cell survival and maturation, and electrophysiological activity. The positive effects of ESC-pMNs on ESC-V2a axon growth and maturation could be enhanced with culture in optimized concentrations of GDNF and PDGF, which act specifically on ESC-pMNs but not ESC-V2a INs. While the model presented herein is simplistic, there is ample opportunity to add or alter cell types, substrates, and pharmacological agents to generate complexity, which can ultimately result in better-informed combinatorial transplantation strategies.

3.2 Introduction

Attempts to reverse the deficits incurred after spinal cord injury are stymied by the multitude of physical and chemical barriers that limit the capacity of the central nervous system (CNS) for regeneration. These problems have motivated the investigation of combinatorial therapies that have the potential to synergistically overcome barriers to functional recovery. Cell transplantation and the delivery of pro-regenerative factors are two major strategies that have been effective. Following the initial trauma, a secondary cascade of events results in the widespread death of local cell populations, but also triggers the upregulation of growth factors and receptors to bolster cell survival 24-48 hours post-injury (Bareyre and Schwab 2003; Di Giovanni et al. 2003). Many cell types and growth factor cocktails have been tested in animal models of SCI to enhance regeneration (Tetzlaff et al. 2011; Thuret et al. 2006). These include stem cell-derived neuronal and glial populations, as well as neurotrophins such as glial-cell line
derived neurotrophic factor (GDNF) (Blesch and Tuszynski 2003; Deng et al.; Zhang et al.),
neurotrophin 3 (NT-3) (Bregman et al.; Cao 2005; Grill et al. 1997; Lu et al.; Mitsui et al. 2005;
Namiki et al. 2000; Nothias 2005; Schnell et al. 1994; Shumsky 2003; Taylor et al. 2006a;
Tobias 2003; Tuszynski 2003; Zhou et al. 2003; Zhou and Shine 2003), brain-derived
neurotrophic factor (BDNF) (Bregman et al.; Cao 2005; Jakeman et al. 1998; Jin et al. 2002; Jin
Nothias 2005; Shumsky 2003; Tobias 2003; Weishaupt et al.; Ye and Houle 1997), and platelet-
derived neurotrophic factor (PDGF) (Almad et al. 2011; Ijichi et al. 1996; Johnson et al. 2010c;
McCreedy et al. 2014b; Wilems et al. 2015). More recent work has centered around the co-
delivery of these factors with cells, either in combinatorial scaffold systems (Johnson et al.
2010c; Lu and Tuszynski 2008; Lu et al. 2012b; McCreedy et al. 2014b; Straley et al. 2010;
Wilems et al. 2015) or by using genetic modifications for sustained delivery within host tissue
(Blits and Bunge 2006; Koda 2004; Taylor et al. 2006a) or the transplanted population (Blesch
and Tuszynski; Cao 2005; Grill et al. 1997; Ijichi et al.; Jin et al. 2002; Lu et al.; Lu et al.
2005; Mitsui et al. 2005; Shumsky 2003; Tobias 2003). Improved cell survival, remyelination,
neuronal differentiation and regeneration are some of the benefits of such combination therapies;
however, little research has been done to investigate whether specific spinal neurons are
differentially affected by growth factor treatment, and if so, how to optimize treatment
combinations to target populations required for functional recovery. Not only are genetically
defined subpopulations of spinal neurons in adult animals difficult to identify/isolate, but such
optimization studies in classic rodent models of SCI are impracticable. Conversely, study of
peripheral nervous system (PNS) neuropathies has benefitted from the wide use of dorsal root
ganglion (DRG) to answer questions in vitro regarding the molecular mechanisms of disease and degeneration, and as a tool for pharmacological screening (Melli and Hoke 2009). We endeavored to develop a similar in vitro model to investigate spinal interneuron (IN) populations that may be differentially involved in rewiring events following SCI.

Evidence from a range of animal models suggests that local INs contribute to functional recovery by bridging the injury site and serving as relays in novel circuits formed between intact spinal segments (Bareyre et al. 2004; Courtine et al. 2009; Courtine et al. 2008; Lang et al. 2012). Cell replacement strategies using INs have the potential to induce recovery via the same rewiring mechanisms, and with endogenous recovery, may significantly improve locomotion after SCI (Conta and Stelzner). A major impediment to the investigation of these populations has been source material—because INs are primarily identified by their developmental transcription factor profiles, isolation required cell sorting of transgenic mouse embryos. Not only are such methods expensive and time-consuming, but a single IN population constitutes only a small fraction of spinal cells. We recently demonstrated an efficient method to differentiate and enrich several spinal populations from transgenic mouse embryonic stem cells (ESCs) using antibiotic selection. By expressing puromycin resistance under the control of a developmental transcription factor of interest, exposure to puromycin in induced cultures eliminates all but the cell type desired, including remnant ESCs capable of teratoma formation. Our lab has thus far been able to isolate large quantities of progenitor motor neurons (pMNs), motor neurons (MNs), V2a INs and V3 INs using this method for in vitro modeling and transplantation (Iyer et al. 2016a; McCreedy et al. 2014a; McCreedy et al. 2012; Xu et al. 2015).
Here we report an aggregate culture platform to study specific ESC-derived spinal IN types in response to growth factor exposure and co-culture conditions. By culturing these populations modularly as spherical aggregates that mimic DRG or neurospheres, we attempted to create a cellular microenvironment closer to that in vivo, while simultaneously allowing for the application of well established metrics to measure growth response. Although the methods herein can be applied to a variety of spinal types generated by our lab and others, we used ESC-derived V2a INs (ESC-V2as) and pMNs (ESC-pMNs) as candidate populations. A combination of morphological and molecular analyses was applied to determine whether the application of neurotrophins typically delivered after SCI (NT-3, GDNF, BDNF, and PDGF) enhanced neurite outgrowth and maturation.

3.3 Methods

3.3.1 Stem Cell Culture

Transgenic and RW4 mouse ESCs were cultured on gelatin-coated T-25 flasks in complete media, consisting of Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies #11965-092, Carlsbad, CA) containing 10% newborn calf serum (Life Technologies #16010-159), 10% fetal bovine serum (Life Technologies #26140-079), and 1x Embryomax Nucleosides (Millipore #ES-008-D, San Francisco, CA). ESCs were passaged every two days at a 1:5 ratio in fresh complete media containing 1000 U/mL leukemia inhibitory factor (LIF; Millipore #ESG1106) and 100µM β-mercaptoethanol (BME; Life Technologies #21985-023). Chx10-Puro ESCs constitutively expressing a fluorescent TdTomato marker were used to obtain ESC-V2as; ESC-pMNs were obtained using non-fluorescent Olig2-Puro ESCs.
3.3.2 Neural Induction and Selection

All cell lines were differentiated using a “2/4+” induction protocol (Figure 3.1) and selected using puromycin as appropriate; 1 x 10^6 ESCs were seeded on day 0 (d0) into 10 mL DFK5 media on an agar-coated 100 mm petri dish for two days to form embryoid bodies (EBs). For Chx10-Puro cells on d2, the media was replenished with DFK5 containing 10 nM retinoic acid (RA; Sigma #R2625) and 1µM purmorphamine (EMD Millipore #540223); on d4 the media was replaced with DFK5 containing 10nM RA, 1µM purmorphamine and 5µM N-{N-(3,5-difluorophenacetyl-L-alanyl)}-(S)-phenylglycine-t-butyl-ester (DAPT; Sigma #D5942). For Olig2-Puro cells, the media was replenished on d2 and d4 with DFK5 containing 2 µM RA and 500 nM Smoothened agonist (SAG; EMD Millipore #566660); 4 µg/mL puromycin (Sigma #P8833) was added to Olig2-Puro media at d4 for selection.

All cell lines were dissociated for selection following induction; Chx10-Puro cells were re-suspended in a selection media of DFK5NB containing B-27 supplement, glutaMAX (Life Technologies #35050-061), 5 ng/mL GDNF (Peprotech #450-10, Rocky Hill, NJ), 5 ng/mL BDNF (Peprotech #450-02, Rocky Hill, NJ), 5 ng/mL NT-3 (Peprotech #450-03), 5 ng/mL PDGF-AA (Peprotech #100-13A), and 2 or 4 µg/mL puromycin in water. For aggregate formation, differentiated Chx10-Puro were plated on poly-L-ornithine/laminin coated T75 flasks at a density of 5x10^5 cells/cm^2 in the selection media. Differentiated Olig2-Puro cells were resuspended in DFK5 containing 4 µg/mL puromycin in water and plated on poly-L-ornithine coated T75 flasks at a density of 5x10^4 cells/cm^2. For long-term 2D cultures, Chx10-Puro and Olig2-Puro cells were plated at the same densities in poly-L-ornithine/laminin coated 24 well plates.
3.3.3 Aggregate Formation

After 24 hours of puromycin selection, ESC-V2as and ESC-pMNs were washed twice with DMEM/F12 and then lifted from flasks using Accutase (Sigma, #A6964) for 30 min at room temperature. 5 x10^5 cells were seeded into each well of a 400 µm Aggrewell plate (StemCell Technologies, #27845) for 2 days in the appropriate media to form aggregates for transplantation. Mixed groups were seeded at a 1:1 ratio, and half the media was replaced daily. Aggregates were lifted by trituration and allowed to settle in microcentrifuge tubes prior to plating as appropriate.

3.3.4 Maturation Cultures

Cells were cultured for 2 weeks on poly-L-ornithine/laminin coated wells either as aggregates or in 2D for maturation studies. Approximately 40-60 aggregates were plated within each well of a 48-well plate, or induced Chx10-Puro and Olig2-Puro were plated at a density of 5x10^5 cells/cm^2 5x10^4 cells/cm^2 in 24-well plates for selection prior to treatment. The media was replaced with supplemented DFK5NB containing 3 growth factor formulations: a no-growth factor control, a low dose (10ng/mL each of NT3, GDNF, BDNF, and PDGF) or an optimal dose (50 ng/mL NT-3, 200 ng/mL GDNF, 200 ng/mL BDNF, and 50 ng/mL PDGF). The media was switched to supplemented Neurobasal with appropriate growth factors for the subsequent week; half the media was replenished every 3 days.

3.3.5 Neurite Extension Assay

Neuroaggregates were plated sparsely on poly-L-ornithine/laminin coated wells — approximately 20-30 aggregates within each well of a 24-well plate—and were exposed to
varying concentrations of NT-3, GDNF, BDNF, or PDGF in supplemented DFK5NB for 24-48 hrs prior to fixation in 4% paraformaldehyde. Aggregates were imaged using an Olympus IX83 (Olympus) inverted microscope equipped with an ORCA-R2 color charge-coupled (Hamamatsu) camera and MetaMorph software (Molecular Devices) under a 10x objective; images were merged using standard Photoshop scripts. Aggregate sizes and neurite extension were measured using ImageJ software, with at least 15 aggregates measured per biological replicate for neurite extension and at least 150 aggregates measured for size distribution per biological replicate (n=3 biological replicates). Neuroaggregates near the edges of the well or very near adjacent neuroaggregates were avoided when possible. The average neurite extension was calculated as the radius of an annulus between the neuroaggregate body and the outer halo of extending neurites. The data were reported as either the absolute neurite length or the average neurite extension of the experimental group normalized to the average neurite extension of the untreated control from the same time point.

3.3.6 GCamp6f-Mediated Calcium Imaging and Analysis

Calcium imaging was performed in four aggregate groups: ESC-V2as, ESC-pMNs, V2a/pMNs, and ESC-V2a aggregates co-plated with ESC-pMN aggregates. Aggregates were seeded onto poly-L-ornithine/laminin coated wells in supplemented DFK5NB media containing 5ng/mL each of NT-3, GDNF, BDNF, and PDGF, and 0.5uL/mL AAV1-hSyn-GCamp6f (Penn Vector Core) for 24 hours. The media was replaced with supplemented DFK5NB containing varying growth factor concentrations for one week, and then on supplemented Neurobasal with appropriate growth factors for the subsequent week; the media was replenished every 3 days. After 14-16 days, calcium transients were recorded using an Olympus IX83 (Olympus) inverted
microscope equipped with an ORCA-R2 color charge-coupled (Hamamatsu) camera and MetaMorph software (Molecular Devices). An incubation system (model ZILCS) was used to maintain the cultures at 37C for the duration of the time-lapse recordings. Acquisitions using a 10x objective occurred every 250 ms for 750 frames; TIFF stacks were imported into ImageJ to obtain Z-axis profiles for 5-10 regions of interest (ROI). A MATLAB script was used to calculate the $\Delta F/F_0$; the background fluorescence was subtracted from the average ROI intensity, and then the ROI was normalized to the average baseline fluorescence. Calcium transients were plotted as $\Delta F/F_0$ versus time.

3.3.7 Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, then permeabilized with 0.1% Triton-X 100 in PBS. After blocking with 5% normal goat serum (NGS), the samples were incubated in the primary mouse anti-neurofilament antibody (1:100, DSHB #SV2) in 2% NGS overnight at 4C. After washing 2 times, the samples were incubated at room temperature with secondary Alexa Fluor conjugated goat antibodies (1:200, Life Technologies) in 2% NGS for one hour. Nuclei were counterstained with Hoechst (1:1000, Life Technologies #H3569).

3.3.8 qRT-PCR

RNA was extracted using RNEasy Mini-kit (Qiagen) directly after aggregation formation or after two weeks in culture as required. 100 ng RNA was used for each cDNA reaction; cDNA was synthesized using a High Capacity RNA-to cDNA Kit (Invitrogen), then used with TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA) and TaqMan Fast Advanced Master Mix (Applied Biosystems) as recommended. qRT-PCR was performed using a Step One
Plus Applied Biosystems thermocycler with the following protocol: 95°C for 20s; 40 cycles of 95°C for 1s and 60°C for 20s. There were n=3 technical replicates completed per run and n≥3 biological replicates for each condition. The comparative ΔCt method was used to analyze expression levels; target genes were normalized against β-actin levels. All statistical analyses were performed using ΔCt values. The data are reported in fold changes \((2^{-\Delta\Delta Ct})\) of relative mRNA expression, where undifferentiated ESCs were used as a comparative control.

### 3.3.9 Statistical Analysis

Statistical analyses were performed using Statistica software (v5.5, StatSoft, Tulsa, OK). Significance was determined using Scheffe’s post hoc test for analysis of variance (ANOVA) with 95% confidence or greater. Average values reported with error bars are the standard deviation unless otherwise indicated.
3.4 Results

3.4.1 Enriched ESC-Derived Neurons form Homogenous Neuroaggregates

To generate homogenous aggregates of ESC-derived spinal cells, we first used two transgenic ESC lines previously developed in the lab—the Chx10-Puro and Olig2-Puro lines—to generate large quantities of ESC-V2as and ESC-pMNs in isolation. ESCs were cultured as EBs for 2 days, then exposed to a combination of RA, a Sonic hedgehog agonist (SAG or purmorphamine), and/or the Notch inhibitor DAPT for 4 days to enrich for the appropriate
population (Figure 3.1A). The EBs were dissociated and plated into flasks for selection with puromycin for 24 hours. Purified cells were then lifted, quantified, and seeded into commercially available Aggrewell dishes alone or mixed at a 1:1 ratio (Figure 3.1B,C); over the course of 48 hours, the ESC-V2as and ESC-pMNs form stable neuroaggregates that exhibit interpenetrating neurite networks (Figure 3.1D,E). By altering the seeding density, the size distribution of neuroaggregates could be tightly controlled; with an initial seeding density of 500,000 cells per well, we obtained ESC-V2a and V2a/pMN neuroaggregates measuring 131.68 ± 19.47 µm and 143.67 ± 22.16 µm in diameter respectively.

In order to confirm that the neuroaggregates would consistently extend processes when plated, ESC-V2a or V2a/pMN neuroaggregates were seeded sparsely on laminin-coated plates in the absence of growth factors, and imaged after 24 or 48 hours for quantification of neurite extension. ESC-V2a extension was significantly greater at both time points in the V2a/pMN neuroaggregates compared to ESC-V2a neuroaggregates alone (Figure 3.2A) \((p<0.0001)\). Morphological differences between the groups were also apparent; ESC-V2a neuroaggregates demonstrated more fasciculate neurite morphology than the mixed group (Figure 3.2 B, C). By 24 hours, non-fluorescent ESC-pMN cells were observed migrating alongside ESC-V2a axons, resulting in a more fragmented body compared to the highly defined spherical bodies observed in ESC-V2a neuroaggregates (Figure 3.2D-G).
Figure 3.2: Morphological differences between ESC-V2a and V2a/pMN neuroaggregates. (A) Average neurite extension of TdTomato+ ESC-V2a neurites after 24 or 48 hours in the absence of growth factors (n=3). Error bars shown as SEM. ***p<0.001 compared to ESC-V2a neuroaggregates at the same time point. Representative fluorescent images of (B) ESC-V2a and (C) V2a/pMN neuroaggregates aggregates grown on laminin for 24 hours in the absence of growth factors. TdTomato+ neurites from V2a/pMN have a more loosely arranged morphology and extend farther from the neuroaggregate body (Scale bars = 100 µm) (D-E) ESC-V2a neuroaggregate bodies remain spherical after 24 hours compared to (F-G) V2a/pMN neuroaggregate bodies, which show the outward migration of TdTomato− cells (Scale bars = 200 µm).
3.4.2 ESC-V2a Neurite Extension in Response to Growth Factors and Co-Culture with ESC-pMNs

BDNF, GDNF, NT3, and PDGF are a few growth factors typically investigated for their therapeutic properties after SCI; in order to determine the feasibility of using these factors for our in vitro assay, ESC-V2a, ESC-pMN, and V2a/pMN neuroaggregates were examined for appropriate receptor expression using qRT-PCR. Various tyrosine receptor kinases are responsible for mediating the trophic effects of these growth factors. BDNF and NT3 operate with the Trk family of receptors, with specific affinity to TrkB and TrkC respectively, but some cross-reactivity occurs (Barbacid 1994; Barbacid 1995; Binder and Scharfman 2004; Maisonpierre et al. 1990). GDNF binds to GDNF receptor α-1 (GDNFR), which in turn activates c-Ret receptor tyrosine kinase (Airaksinen and Saarma 2002; Baloh et al. 2000). PDGFR-α is the predominant PDGF receptor found in the CNS, and though commonly associated with oligodendrocyte progenitors, it is also expressed in a variety of neuronal cells including MNs (Andrae et al. 2008; Calver et al. 1998; Rivers et al. 2008).
Figure 3.3: Differential growth factor receptor expression in ESC-V2as and ESC-pMNs. qRT-PCR results of neuroaggregates collected at d2 prior to plating (n=3); data reported as expression fold change of (A) TrkB, (B) TrkC, (C) GDNFRα1, and (D) PDGFR compared to undifferentiated ESC control. The green and red dotted lines correspond to upregulation or downregulation, defined by a 2-fold difference in gene expression compared to undifferentiated ESC controls. Error bars shown are the standard deviation. *p<0.01 compared to ESC-V2a neuroaggregates.
All groups demonstrated significant upregulation of TrkB and TrkC compared to undifferentiated ESC controls \((p<0.001)\); there were no significant differences in Trk expression between the groups (Figure 3.3A,B). ESC-pMNs and V2a/pMNs exhibited significantly greater expression of GDNFR than the ESC control \((p<0.01)\), with ESC-pMNs also demonstrating significantly greater GDNFR expression than ESC-V2as \((p<0.01)\) (Figure 3.3C). Similarly, ESC-pMNs showed significantly greater PDGFR expression than either ESC-V2as or the ESC control \((p<0.01)\) (Figure 3.3D). GDNFR appeared slightly upregulated in V2a INs compared to the undifferentiated control, but PDGFR not expressed in ESC-V2as (Figure 3.3C,D). Expression of both GDNFR and PDGFR was upregulated in the V2a/pMN group, but not significantly so compared to ESC-V2as, as might be expected in a mixed population. These data suggest that our selected growth factors are valid choices for a quantitative assessment of the growth factor dose response on ESC-V2a neurite extension when alone or when co-cultured with ESC-pMNs.
Figure 3.4: Neurite outgrowth of ESC-V2a neuroaggregates in vitro in response to different growth factors concentrations. The average neurite extension and neurite extension normalized to the average no-treatment control condition for experimental groups treated with (A,B) BDNF, (C,D) GDNF, (E,F) NT3, and (G,H) PDGF. Although neurite length increased between 24 and 48 hours, the normalized extension compared to the untreated control was the same or reduced in all conditions by the 48 hours timepoint. Error bars shown are the SEM. *p<0.01, #p<0.05 compared to untreated controls.

ESC-V2a and V2a/pMN neuroaggregates were plated sparsely on laminin-coated wells and exposed to BDNF, GDNF, NT3, and PDGF at concentrations ranging from 12.5-200 ng/mL. Images were taken at 24 hours and 48 hours; we calculated both the absolute neurite extension as well as the neurite extension of the experimental group normalized to the average neurite extension of the no-treatment control. As previously shown (Figure 3.2), co-culture with pMNs resulted in significant enhancement of ESC-V2a neurite extension in the absence of growth factors.
factors; this trend persisted with growth factor treatment (Figure 3.4, Figure 3.5). Although growth factor-treated ESC-V2a neuroaggregates showed significant differences in neurite extension compared to untreated controls at one or more concentrations at 24 hours, demonstrable of a biphasic response, these effects were mitigated by 48 hours, resulting in no change or a significant reduction in outgrowth compared to untreated controls (Figure 3.4). When cultured in the presence of ESC-pMNs, optimal doses of BDNF, GDNF, NT3 and PDGF amplify the positive role of co-culture (Figure 3.5). At 48 hours, a concentration of 200 ng/mL BDNF, 200 ng/mL GDNF, or 50 ng/mL NT3 result in significantly greater ESC-V2a neurite extension compared to the untreated control. Although 50 ng/mL PDGF results in significant extension at 48 hours, this effect is mitigated by 48 hours. From these observations, an optimum combination dose of 50 ng/mL NT3, 200 ng/mL GDNF, 200ng/mL BDNF, and 50ng/mL PDGF was selected for maturation studies.
Figure 3.5: Neurite outgrowth of V2a/pMN neuroaggregates in vitro in response to different growth factors concentrations. The average neurite extension and neurite extension normalized to the average no-treatment control condition for experimental groups treated with (A,B) BDNF, (C,D) GDNF, (E,F) NT3, and (G,H) PDGF. Significant neurite extension was observed at discrete growth factor concentrations: 200 ng/mL BDNF, 200 ng/mL GDNF, 50 ng/mL NT3, and 50 ng/mL PDGF. Error bars shown are the SEM. *p<0.01, #p<0.05 compared to untreated controls.
Figure 3.6: Immunocytochemistry analysis of neuroaggregate integrity after two weeks in culture. ESC-V2a (A-F), V2a/pMN (G-L), and ESC-pMN neuroaggregates cultured for two weeks in the absence of growth factors (Control; A-B, G-H, M-N), with a low dose of growth factors (10 ng/mL each) (C-D, I-J, O-P), an optimal dose (50 ng/mL NT3, 200 ng/mL GDNF, 200 ng/mL BDNF, and 50 ng/mL PDGF) (E-F, K-L,Q-R). Cultures were stained with the nuclear marker Hoechst (blue) and NF (green); ESC-V2a neurites are marked with TdTomato (red) (Scale bars = 100 µm). All ESC-V2a groups show degradation of neurites. V2a/pMN and ESC-pMN groups show enhanced NF staining with higher growth factor concentrations, as well as a greater quantity of cell nuclei migrating from the body. (S) Representative image of glia migrating from neuroaggregate (Scale bar = 100 µm) and (T) wrapping processes around NF+ axons (Scale bars = 50 µm).
3.4.3 Effect of Optimized Growth Factor Dose on ESC-V2a and ESC-pMN Maturation

To determine the role that an optimized dose of growth factors might have on culture maturation, ESC-V2a, ESC-pMN, and V2a/pMN neuroaggregates were cultured for two weeks in media containing no growth factor, a low dose of 10 ng/mL per growth factor, or media containing the optimum combination. Aggregates were stained for the mature neuronal maker neurofilament (NF) via immunocytochemistry to assess the integrity of surviving neurons (Figure 3.6). After 2 weeks, ESC-V2a neuroaggregates in all conditions demonstrated dramatic axon degeneration; long axon trails indicate substantial axonal extension prior to die back. By comparison, NF\(^+\) axons in both ESC-pMN and V2a/pMN groups remained intact and robust, and the quantity of NF\(^+\) cells was greater in both treatment groups compared to the no-treatment controls. The improvement in neuronal viability can be attributed to the proliferation of glial cells in both ESC-pMN and V2a/pMN cultures. TdTomato\(^-\) cell bodies with oligodendrocyte-like morphologies radiate from the neuroaggregate bodies and cluster around NF\(^+\) and TdTomato\(^+\) axons. The density of glia is greater than in treatment cultures than in no-treatment controls, as evidenced by the higher quantity of nuclei that have migrated from the aggregate body.
Figure 3.7: Expression of maturation markers after two weeks in culture as neuroaggregates. qRT-PCR results of (A) SV2, (B) Vglut2, and (C) ChAT from neuroaggregates collected at d14 (n=3); data reported as expression fold change compared to undifferentiated ESC control. V2a/pMN and ESC-pMN groups were exposed to low doses of growth factors (10 ng/mL each), an optimal dose (50 ng/mL NT3, 200 ng/mL GDNF, 200 ng/mL BDNF, and 50 ng/mL PDGF), or were cultured in the absence of growth factors (Control). Error bars shown are the standard deviation. The green and red dotted lines correspond to upregulation or downregulation, defined by a 2-fold difference in gene expression compared to undifferentiated ESC controls. *p<0.05 compared to V2a/pMN control. †p<0.05 compared to ESC-pMN control.

The relative mRNA expression for choline acetyl transferase (ChAT), vesicular glutamate transporter 2 (Vglut2) and synaptic vesicular marker 2 (SV2) was measured to examine the maturation of MNs and V2a INs, as well as the relative quantity of synapse formation in ESC-pMN and V2a/pMN cultures (Figure 3.7). Vglut2 and SV2 were significantly upregulated in all treatment groups and the V2a/pMN control compared to the undifferentiated ESC control (p<0.001) and the ESC-pMN no-treatment control (p<0.01) (Figure 3.7A,B). SV2 was significantly expressed in the no-treatment V2a/Olig2 control compared to ESC-pMN control. Treatment with either low or optimized doses also significantly enhanced SV2 expression in V2a/Olig2 cultures compared to the no-treatment control (Figure 3.7A). Similarly,
ChAT was significantly upregulated in all treatment groups compared to the undifferentiated ESC control as well as the no-treatment controls (p<0.001); there were no significant differences between treatment groups (Figure 3.7C).

Figure 3.8: Expression of maturation markers after two weeks in 2D culture. qRT-PCR results of (A) SV2, (B) Vglut2, and (C) ChAT from ESC-V2a and ESC-pMN 2D cultures collected at d14 (n=2); data reported as expression fold change compared to undifferentiated ESC control. V2a/pMN and ESC-pMN groups were exposed to low doses of growth factors (10 ng/mL each), an optimal dose (50 ng/mL NT3, 200 ng/mL GDNF, 200 ng/mL BDNF, and 50 ng/mL PDGF), or were cultured in the at the optimal dose of only a single growth factor. Immunocytochemistry staining for the nuclear marker Hoechst (blue) and NF (green) show lower quantities of mature neurons in ESC-pMN cultures treated with the low dose of growth factors (D-F) compared to the optimal dose (G-I).
Because the viability of ESC-V2a neuroaggregates was critically low after two weeks in culture, ESC-V2as and ESC-pMNs were plated in 2D culture to investigate the role that the single growth factor doses might have on maturation compared to the combination treatments. The data reported is a preliminary study of mRNA expression for maturation markers with only two biological replicates, so while no statistically significant claims can be made, some trends are apparent. Though treatment of ESC-V2as with PDGF alone appears to cause a reduction in Vglut2 expression, there are no other appreciable trends in SV2 or Vglut2 expression levels in the other groups. Conversely, SV2 expression levels trend upward in ESC-pMN cultures in combination groups compared to single treatment groups. The most striking effect is on ChAT expression; while treatment of PDGF alone reduces ChAT expression, treatment with GDNF alone improves expression compared to the other single treatment groups and the low dose combination treatment. This improvement is enhanced in the optimized treatment; a comparison of NF staining in low dose versus optimized cultures shows greater axonal staining congruent with an increase in ChAT expression. Together, these initial data suggest that while these growth factors do not affect ESC-V2as in isolation, they can stimulate ESC-pMN maturation, which may in turn have positive trophic effects on ESC-V2a that improve neurite extension and viability.
Figure 3.9: Calcium imaging from neuroaggregates after 2 weeks. Representative neuroaggregates transduced with GCaMP6f at (A) baseline and (B) during spiking; color map corresponds to fluorescence intensity. Calcium fluorescence intensity for (C) V2a/pMNs, (D) ESC-pMNs, and (E) ESC-V2as co-plated with ESC-pMNs is reported as the change in fluorescence normalized to baseline for a 3 minute recording period.

3.4.4 Calcium Activity in Neuroaggregates in Response to Growth Factors

While no significant differences in gene expression were observed in optimized aggregate cultures compared to low dose cultures, we wanted to determine whether the growth factor treatments altered neuronal activity. In addition to V2a/pMN, ESC-pMN, and ESC-V2a groups, ESC-V2as were co-plated with ESC-pMNs (ESC-V2a/ESC-pMN) to determine whether ESC-V2a integrity and activity could be rescued by proximity to ESC-pMNs. All
neuroaggregates were transduced with an adeno-associated virus encoding GCamp6f under the synapsin promoter, which allowed for visualization of calcium transients in neuronal populations (Chen et al. 2013b) (Figure 3.9A,B). Recordings of spontaneous activity were taken after 2 weeks in culture. Robust activity was observed in V2a/pMN, ESC-pMN, and ESC-V2a/ESC-pMN groups. When cultured in the absence of growth factors, all groups demonstrated reduced, arrhythmic behavior compared to groups treated with growth factors (Figure 3.9C-E). V2a/pMN cultures showed improved rhythmicity and greater bursting with the optimal treatment compared to the low dose of growth factors (Figure 3.9C). Conversely, ESC-pMNs showed more regular activity at the low dose than the optimal dose (Figure 3.9D), which followed trends in SV2 expression observed via qRT-PCR (Figure 3.8A). Calcium transients in ESC-pMN culture were primarily single spikes, compared to the 2-4 spike bursts observed in V2a/pMN neuroaggregates (Figure 3.9C,D). Furthermore, a small population of neurons in ESC-pMN cultures fired asynchronously, compared to the high level of synchronicity observed in mixed cultures containing ESC-V2as (Figure 3.9D-E). No calcium activity was observed in ESC-V2a neuroaggregates cultured alone (data not shown), consistent with the degradation of axonal processes (Figure 3.6A-F). ESC-V2as survived and matured when co-cultured with ESC-pMNs; with growth factor treatment, neuroaggregates were capable of rhythmic multi-spike bursting, which was enhanced in the optimal dose condition as in V2a/pMN cultures (Figure 3.9E). These data suggest that optimizing growth factor and co-culture conditions can improve the maturity of neuronal activity in ESC-V2as.
3.5 Discussion

3.5.1 Co-culturing ESC-V2as and ESC-pMNs as a Modular In Vitro System for CNS Drug Screening

The in vitro model developed herein allows us to perform high throughput pharmacological screens in spinal neurons; we used mouse ESC-derived V2a INs and pMNs as model populations because both are strong candidates for transplantation and as targets in endogenous host tissue (Iyer et al. 2016b). V2a INs form an important component of the mammalian central pattern generator, and are involved in coordinating left-right alternation, skilled-reaching, and rhythmic breathing (Azim et al. 2014; Crone et al. 2008; Crone et al. 2012; Crone et al. 2009; Dougherty and Kiehn 2010a; Zhong et al. 2010; Zhong et al. 2011). The pMN population gives rise to MNs, oligodendrocytes, and astrocytes during development (Jessell 2000; Richardson et al. 2000). MNs are an attractive population for transplantation because they receive input from various IN populations within the cord, including V2a INs, and are directly responsible for downstream muscle innervation (Davis-Dusenbery et al. 2014; Dougherty and Kiehn 2010b; Nogradi et al. 2011; Tanabe et al. 1998b); oligodendrocytes and astrocytes provide myelination and trophic support critical for neuronal survival and the formation of new functional circuits (Brustle et al. 1999; Clarke and Barres 2013; Keirstead et al. 2005; Sharp et al. 2010; Zhang et al. 2006).

In culturing these ESC-derived populations modularly as spherical aggregates, we attempted to recapitulate DRG explant cultures, which have been used since the late 1970s as a tool for drug screening to optimize regeneration and myelination in the PNS (Eldridge et al.; Melli and Hoke 2009; Scott; Wood and Bunge). Compared to monolayer cultures, 3D and
aggregate cultures demonstrate behaviors and properties more closely related to those seen in vivo (Edmondson et al. 2014; Lu et al. 2012a; Pampaloni et al. 2007). That our selected ESC-derived populations would form stable neuroaggregates was not intuitive, especially for ESC-V2as, which did not have the physical support that glia provide. Furthermore, the size of neuroaggregates could be tightly controlled by changing the seeding density of selected ESC-derived populations into aggregate wells. We chose a small aggregate size to avoid viability issues associated with toxic waste buildup caused by poor vascularity. The addition of other cell types is also possible; by titrating in populations of interest to optimal ratios, neuroaggregates that present with ideal molecular and electrophysiological properties can be generated. Culturing populations as neuroaggregates prior to transplantation may also improve the survival of post-mitotic neurons compared to typical single-cell suspensions; cells in the neuroaggregate appear to form a supportive mesh structure, which could provide a buffer against the stress induced by the SCI environment. Indeed, as discussed in Chapter 4, transplanted neuroaggregates survive injection into a cervical spinal cord lesion without the benefit of growth factors or a biomaterials scaffold.

3.5.2 ESC-V2as Demonstrate Muted Response to Selected Growth Factors in the Absence of ESC-pMNs

Using this platform, we wanted to test the hypothesis that V2a IN axon growth could be stimulated in a dose-dependent manner by NT3, GDNF, BDNF, and PDGF, and/or by co-culture with pMNs. Although neurite extension studies in the ESC-V2a neuroaggregates suggest a biphasic response at 24 hours, whereby there are significant improvements in outgrowth at discrete concentrations compared to the untreated control, by 48 hours, treatment groups were
comparable or significantly worse than the control group. There are several explanations for these observations that could be a result of experimental design flaws: first, that the growth factor concentrations tested did not encompass the optimal range for this particular cell type. *In vivo* studies often use growth factor doses that are a magnitude or more higher than those used here (Lu *et al*. 2012b), though the range used for *in vitro* studies can be lower because metabolic clearance is negligible. Alternatively, the optimal dose was within the tested range, but the effectiveness occurred over a very tight distribution of concentrations, which we bypassed with our step size. Saturation of the receptors also could have caused the variable or reduced extension in treated groups compared to the control. An important consideration is the time point at which these neuroaggregates were tested. Because the ESC-V2a INs used in the aggregates were still immature, the effects observed may not be reflective of the response of mature ESC-derived or endogenous V2a IN populations. If this is the case, then the delivery of growth factors alongside these ESC-derived IN populations for transplantation studies may not have a significant effect on cells within the graft, but may still affect host populations. It might also suggest that delaying growth factor delivery to the transplant and/or ensuring that the delivery is sustained through the maturation of those populations are more efficacious strategies. By aging the neuroaggregates in suspension culture prior to seeding for the neurite extension assay, maturity-dependent effects could be parsed. It should be noted that at time points beyond 48 hours, neurites began to curl erratically and more regularly started to interact with other neuroaggregates in the same well (data not shown). Because of these factors, the neuroaggregates at the size and density chosen for this study were no longer suitable for consistent, repeatable neurite extension measurements. Altering the experimental parameters
would allow for longer periods of study, which may be required to discern long-term differences between growth factor treatments.

Notably, the addition of growth factors did not prevent the degeneration of these neurons in the two-week maturation studies *in vitro*. Glutamate toxicity in these artificial, isolate cultures could be the culprit. If growth factors indeed have no effect on long-term V2a survival and plasticity, there could be far-reaching implications for functional regeneration, since studies of partial SCIs where recovery is possible suggest that remodeling of local INs about the injury site is responsible for locomotor gains (Bareyre *et al.* 2004; Courtine *et al.* 2009; Courtine *et al.* 2008). As a general caveat, however, *in vitro* models are typically designed to detect simple cause-effect relationships; there are numerous variables *in vivo* and in mature adult neurons that differ from the *in vitro* mono-culture and co-culture conditions described which prevent defensible conclusions to be made in the absence of clear *in vivo* evidence.

### 3.5.3 Dose-Dependent Response of ESC-pMNs to GDNF and PDGF Improves ESC-V2a Growth and Survival

Co-culture of ESC-V2as with ESC-pMNs in the V2a/pMN conditions significantly improved neurite extension compared to ESC-V2a neuroaggregates alone, even in the absence of growth factors. ESC-V2a neurites had significantly greater extensions when the neuroaggregates were treated with or 200 ng/mL BDNF, 200 ng/mL GDNF, 50 ng/mL NT3, or 50 ng/mL PDGF. The distinct improvements observed in GDNF and PDGF conditions is notable because ESC-V2as do not express GDNFR or PDGFR. This could indicate some kind of dose-dependent response in ESC-pMNs, which indirectly bolstered the ESC-V2a neurite outgrowth. The optimization studies confirm this: ESC-V2a neuroaggregates cultured alone showed dramatic
degeneration in all conditions, but ESC-pMN and V2a/pMN groups were able to survive and differentiate into mature NF+ neurons. This robust survival can be attributed to the trophic support provided by glia in the culture; after 24 hours, ESC-pMN-derived cells begin to migrate from the neuroaggregate body. By two weeks, cells with oligodendrocyte-like morphology were tiled along axons throughout the culture dish; the optimal dose conditions showed greater proliferation of these cells than the low dose or control group. This effect may have been induced by the higher concentration of PDGF, which normally stimulates the proliferation and maturation of immature oligodendrocytes (Calver et al. 1998; Hart et al.; Hu et al. 2008). Because oligodendrocytes provide both trophic support and a permissive substrate for neurons, transplantation of oligodendrocyte progenitor cells has been widely successful, and is now in human clinical trials (Keirstead et al. 2005; Sharp et al. 2010; Zhang et al. 2006). Whether

A preliminary study examining the effect of single growth factor treatments versus combined treatment on maturation in 2D ESC-V2a and ESC-pMN cultures was performed to understand what contributions individual factors had on maturation. Similar to the neurite extension studies, the growth factors seemed to have no effect on the expression of maturation markers in ESC-V2a cultures. However, in ESC-pMN cultures, treatment with 200 ng/mL GDNF increased ChAT expression, which was amplified in the optimal combination condition. Staining confirmed higher levels of NF in the optimal cultures compared to low dose cultures, which was not apparent in the neuroaggregates stained at the same time point; this latter point implies that neuroaggregate culture can mask or alter dynamics seen in typical adherent culture. These findings are in agreement with previous literature demonstrating that GDNF is a potent survival factor for MNs (Henderson et al. 1994; Leitner et al. 1999), but may not be able to
rescue some downstream MN synapses (Suzuki et al. 2007). The optimal dose also resulted in lower expression of Vglut2 in ESC-pMN cultures. Although MNs form cholinergic synapses onto muscles in vivo, in culture and in vitro, they have been found to express Vglut2 and engage in glutamatergic synapses with IN populations. Reduced Vglut2 expression can improve MN viability by decreasing degeneration (Herzog et al. 2004; Wootz et al. 2010). However, a recent study showed that increased glutamatergic receptor expression in phrenic MNs was associated with an improvement in spontaneous functional recovery following a C2 hemisection, and further, that this improvement could be enhanced by forced expression of TrkB (Gransee et al.). The role of glutamatergic receptors in MN plasticity thus appears variable depending on environmental conditions. All together, these data suggest that GDNF and PDGF improve MN and oligodendrocyte differentiation and survival, which are able to indirectly improve the survival of ESC-V2as when co-cultured.

3.5.4 Growth Factor Treatment Enhances Calcium Activity

Using calcium imaging, we observed that treatment with growth factors was able to increase the spiking frequency and burst rate in ESC-pMN and V2a/pMN neuroaggregates compared to no-treatment, and that an optimal growth factor dose may further improve the maturity of firing compared to the low dose condition. Though there was no activity in ESC-V2a neuroaggregates plated alone—consistent with neuronal degradation—plating ESC-V2a neuroaggregates with ESC-pMN neuroaggregates was able to rescue activity in the ESC-V2a group. Indeed, with the optimal treatment, ESC-V2a/ESC-pMN cultures demonstrated comparatively high frequency activity, with bursts between 2-4 spikes, compared to neuroaggregates from the V2a/pMN condition. The difference in frequency and maturity may be
due to differences in the distribution of ESC-V2as on the plate. Unpublished work from the Pfaff lab suggests that altering the ratio of inhibitory and excitatory neurons in ESC-derived IN-MN cultures can dramatically change firing frequency and patterns of activity (Pfaff 2016). It is of note that ESC-derived neurons are still relatively immature at the 2 week time point evaluated here; significant changes in the network structure and firing properties occur between 2-4 weeks in culture (Illes et al. 2007). Similar reorganization on these long time-scales is seen in IN populations in vivo; V3 IN networks undergo alterations up to 3 weeks after birth in post-natal mice (Borowska et al. 2015). The true effect of growth factor treatment on the maturation of these networks likely requires culture up to a month or more.

3.6 Conclusions

The platform described in this report represents a novel opportunity to study and optimize the culture of isolated post-mitotic spinal populations suitable for transplantation. In the absence of supportive ESC-pMNs, ESC-V2as do not respond to growth factor treatment and undergo degeneration within 2 weeks. However, co-culture in optimized conditions enables the maturation and proliferation of neurons and glia in pMN populations, which in turn have an indirect, positive effect on ESC-V2ai integrity and activity. By increasing the complexity of this platform in future studies to include other cell types, substrates, and pharmacological agents, we can work to intelligently refine combinatorial transplantation strategies as well as begin to answer questions regarding the mechanisms behind degeneration and regeneration in spinal IN populations critical for locomotor function.
Chapter 4

Survival and Integration of Enriched Mouse Embryonic Stem Cell-Derived V2a Interneurons Co-Transplanted with Progenitor Populations after Cervical Spinal Cord Injury

4.1 Abstract

Although spinal interneurons are an enticing population to target for cell replacement strategies, transplantation of specific, post-mitotic neuronal populations following spinal cord injury (SCI) has been historically hampered by poor survival coupled with limited source material. While progenitor populations demonstrate a greater potential for regeneration following SCI, there remains significant variability in directing the differentiation of these populations in vivo. With the recent development of a protocol to obtain large quantities of high purity V2a interneurons (INs) from mouse embryonic stem cells (ESCs) in vitro, we sought to determine the feasibility and efficacy of transplanting neural progenitor cells (NPCs) or ESC-derived progenitor motor neurons (pMNs) enriched with this post-mitotic population. ESC-V2as, ESC-pMNs, NPCs, V2a/pMN and V2a/NPC groups were injected into a sub-acute C3/C4 lateral contusion model of SCI in rats and evaluated 1 month post-injury. ESC-V2as were able to survive injection into the injury site when co-transplanted with progenitors, and to a lesser extent
when transplanted alone. Furthermore, progenitor populations and ESC-V2as were observed migrating up to 2 mm away from the injury site on the rostrocaudal axis as well as through the ventral roots. However, transplants containing mouse ESC-derived populations underwent significant attrition, and limited maturation of neuronal cells was observed. Despite these shortcomings, diaphragm electromyography recordings indicate that V2a/NPC transplantation improved breathing compared to NPCs alone, ESC-pMN, V2a/pMN, or vehicle-control groups. Despite the failure of retrograde tracing of phrenic circuits to confirm connectivity of the V2a INs into relevant breathing circuits, this behavioral data suggests a positive role for V2a IN transplantation in functional recovery.

4.2 Introduction

Spinal interneurons (INs) are key players in neural plasticity following SCI; evidence from a range of animal models and human clinical studies suggests that they contribute to functional recovery by bridging the injury site and providing relays between intact spinal segments (Bareyre et al. 2004; Courtine et al. 2008), or by adopting new compensatory roles in response to the altered circuit (Flynn et al. 2011; Fouad and Tse 2008; Harkema 2008b; Jankowska 2001; Lane et al. 2009). These qualities make INs an attractive target for cell replacement strategies but isolating therapeutically appropriate populations for transplantation has been a challenge. Neural progenitor cells (NPCs) isolated from fetal tissue or generated from pluripotent stem cells represent an expandable source of glial and neuronal progenitors (Tetzlaff et al. 2011). However, once transplanted, the extent of neurogenesis can vary and is relatively uncontrolled in the absence of morphogens that might direct differentiation into desired post-
mitotic IN populations. Ethical impediments dog the use of fetal tissue. In graft materials derived from induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs), tumor formation is a realistic concern (Johnson et al. 2010b; Khazaei et al. 2014).

We recently described the development of several selectable ESC lines that allow for the enrichment of specific ventral subpopulations following differentiation in vitro (Iyer et al. 2016a; McCreedy et al. 2014a; McCreedy et al. 2012; Xu et al. 2015). By driving puromycin-N-acetyltransferase expression under the defining transcription factor of interest, the addition of antibiotic to the culture media eliminates undesired cell types, including remnant stem cells. In this study, we investigated the efficacy of transplanting post-mitotic ESC-derived V2a INs (ESC-V2as) in a lateral C3/C4 contusion model of SCI, which compromises the fidelity of respiration. V2a INs in the cervical spinal cord and hindbrain normally contribute to forelimb reaching behaviors and breathing rhythm (Azim et al. 2014; Crone et al. 2012). When activated, V2a INs in these regions drive inspiratory accessory respiratory muscle (ARM) activity in adults, and have been implicated in compensatory remodeling during early stages of neurodegeneration in an amyotrophic lateral sclerosis model (Romer et al. 2016). In replenishing local V2a INs at the SCI lesion, the intention is to aid spontaneous respiratory recovery by recruiting endogenous, ipsilateral pre-motor INs that normally provide input to phrenic motor neurons, thereby bolstering the capacity of the spinal cord to compensate for damaged circuits (Hoh et al. 2013; Lane 2011; Lane et al. 2009).
4.3 Methods

4.3.1 Harvest and Culture of NPCs

Frozen NPCs were derived from E13.5 Thy1-GFP rat embryos as previously described (Bonner et al. 2011)(Figure 4.1A-C). Cells were thawed one day prior to aggregation; 3x10^6 NPCs were seeded onto laminin coated T75 flasks and cultured in DMEM/F12 containing 25 mg/mL bovine serum albumin, B-27 supplement (Life Technologies #17504-044), N2 supplement (Life Technologies #17502-048), 10 ng/mL basic fibroblast growth factor (bFGF; Peprotech 450-10, Rocky Hill, NJ), and 20 ng/mL neurotrophin-3 (NT-3; Peprotech #450-03).

4.3.2 Embryonic Stem Cell Culture and Neural Inductions

A selectable Chx10-Puro mouse ESC line that constitutively expressed TdTomato and an Olig2-Puro mouse ESC line that constitutively expressed eGFP were cultured and differentiated as previously described to produce ESC-V2as and ESC-derived progenitor motor neurons (ESC-pMNs) respectively (Figure 4.1D-I) (Iyer et al. 2016a; McCreedy et al. 2014b). Briefly, mouse ESCs were cultured on gelatin-coated T75 flasks in complete media containing 1000 U/mL leukemia inhibitory factor (LIF; Millipore # ESG1106) and 100 µM β-mercaptoethanol (BME; Life Technologies #21985-023). Both cell lines were differentiated using a “2/4+” induction protocol (Figure 4.1); 1 x 10^6 ESCs were seeded on day 0 (d0) into 10 mL of DFK5 media on an agar-coated 100 mm petri dish for two days to form embryoid bodies (EBs). For Chx10-Puro cells on d2, the media was replenished with DFK5 containing 10 nM retinoic acid (RA; Sigma #R2625) and 1 µM purmorphamine (EMD Millipore #540223); on d4 the media was replaced with DFK5 containing 10 nM RA, 1 µM purmorphamine and 5 µM N-\{(3,5-difluorophenacetyl-L-alanyl)-(S)-phenylglycine-t-butyl-ester (DAPT; Sigma #D5942). For
Olig2-Puro cells, the media was replenished on d2 and d4 with DFK5 containing 2 µM RA and 500 nM Smoothened agonist (SAG; EMD Millipore #566660). EBs were dissociated and re-suspended in a selection media of DFK5NB containing B-27 supplement, glutaMAX (Life Technologies #35050-061), 5 ng/mL glial-derived neurotrophic factor (GDNF; Peprotech #450-10, Rocky Hill, NJ), 5 ng/mL brain derived neurotrophic factor (BDNF; Peprotech #450-02, Rocky Hill, NJ), 5 ng/mL NT-3, and 4 µg/mL puromycin in water (Sigma #P8833). Differentiated Chx10-Puro and Olig2-Puro cells were plated on laminin coated T75 flasks at a density of 5x10^5 and 5x10^4 cells/cm^2 respectively in the selection media for 24 hours.

### 4.3.3 Aggregate Preparation

NPCs, ESC-V2as and ESC-pMNs were lifted from laminin-coated flasks using Accutase (Sigma, #A6964) for 30 min at room temperature; 4 x10^5 cells were seeded into each well of a 400 µm AggreWell plate (StemCell Technologies, #27845) for 2 days in the appropriate media to form aggregates for transplantation (Figure 4.1G). Mixed groups were seeded at a 1:1 ratio, and half the media was replaced daily. Aggregates were lifted by trituration and allowed to settle in microcentrifuge tubes. The media was aspirated and the cells washed once with HBSS prior to transplantation or in vitro characterization.

### 4.3.4 In Vitro Characterization

Phase images of the aggregates were captured and evaluated using ImageJ (NIH, Bethesda, MD) software to determine diameter distributions. To obtain fluorescent images, aggregates in suspension were first flattened between glass coverslips; images were captured using a MICROfire camera attached to an Olympus IX70 inverted microscope. To determine cell
viability, aggregates were prepared using ESC lines that did not contain fluorescent reporters; GFP expression in rat NPCs was sufficiently low enough to be thresholded for analysis. Aggregates were incubated in Accutase for 3 hours at room temperature with agitation, then triturated to dissociate. Cells were incubated for 30 minutes in PBS containing calcein-AM and ethidium homodimer-1 (Life Technologies # C481) prior to imaging. Counting was performed in ImageJ with approximately 300 cells counted per replicate (n=3). Fluorescent aggregates were similarly dissociated and quantified to determine the population distribution within mixed groups.

### 4.3.5 Surgical Procedures

All surgical and animal care procedures were approved by the Institutional Animal Care and Use Committee at Drexel Medical School and followed NIH guidelines. Adult female Sprague Dawley rats (n=35) used for this study were anesthetized by injection of xylazine (5-10mg/kg, s.q.) and ketamine (80-120mg/kg, i.p.) for spinal cord injury and transplantation procedures, and isoflurane (5% in O₂ induction, 2% in O₂ maintenance) for tracing procedures. Upon completion of each procedure, anesthesia was reversed via injection of yohimbine (10mg/ml s.q.). Lactated ringers (5mL, s.q.) was injected to prevent dehydration and buprenorphine (0.025mg/kg s.q.) was used as an analgesic.

For spinal cord injury, animals were prepared for surgery as previously described (Fuller et al. 2009; Lane et al. 2009; Lane et al. 2008c). Briefly, a skin incision was made with a No. 15 scalpel blade from the base of the skull to the fifth cervical segment (C5), surrounding musculature was carefully pushed apart and a laminectomy was made at the third cervical segment (C3) and rostral part of C4. Animals received a lateralized contusion injury just caudal
to C3 dorsal horn on the left side of the spinal cord to obtain a C3/4 lateralized injury using the Infinite Horizon Pneumatic Impactor (Precision Systems, Lexington, Kentucky), with the impact force preset to 200 kilodynes and zero dwell time. All animals were intubated and placed on a mechanical ventilator (Small Animal Ventilator, Harvard Apparatus) for one hour. The underlying muscle was sutured in layers and the skin was closed with wound clips.

For transplantation procedures, all animals were anesthetized as described above and the skin and underlying musculature were re-exposed one-week post-injury. A small hole was then made in the dura overlying the epicenter of the injury (visualized by a bruise in the spinal tissue) and aggregates consisting of NPCs (n=2), ESC-V2as (n=2), ESC-pMNs (n=7), ESC-V2as/ESC-pMNs (n=6), or ESC-V2as/NPCs (n=7) were transplanted stereotactically using a gas tight 25uL Hamilton syringe with a custom, 30 gauge needle. Age-matched controls received all of the described surgical procedures, but instead of cellular aggregates, received an equal volume (6uL) of Hanks' Balanced Salt Solution (n=5) into the lesion cavity. The underlying muscle was sutured in layers and the skin was closed with wound clips. Animals were allowed to recover for a month prior to anatomical tracing procedures and terminal electrophysiology recordings.

All animals were immunosuppressed with Cyclosporin A (10 mg/kg, daily s.q.) beginning three days prior to transplantation and throughout the duration of the study.

4.3.6 Anatomical Tracing

A transynaptic, retrograde tracing technique (pseudorabies virus, PRV) was used to assess the host-graft integration within the phrenic circuit, one-month post-transplantation as previously described (Lane et al. 2008c). Briefly, after reaching a surgical plane of isoflourance-induced anesthesia, a laparotomy was performed to expose the diaphragm and a Bartha strain of
PRV was topically applied onto the hemidiaphragm ipsilateral to injury. This retrograde virus is taken up by phrenic motoneurons innervating the diaphragm and is retrogradely transported to the phrenic motoneuron cell bodies in the spinal cord (primary labeling, occurs within 24 hours of application (Lane et al. 2008c)). The virus then is transported transynaptically to infect the entire phrenic circuit over time (including phrenic interneurons, neurons within the ventral respiratory column, and motor cortex if given enough time). The PRV recombinant that was used in this study is PRV273 (8.0-9.9×10^8 pfu/ml) expressing a blue fluorescent protein, which was enhanced with immunohistochemistry as part of tissue processing.

4.3.7 Electrophysiology

Animals were allowed to survive for 72 hours before undergoing terminal, bilateral, diaphragm electromyography (dEMG) as described elsewhere (Lane et al. 2012), just prior to being euthanized and perfusion-fixed for histological analyses. Briefly, animals were anesthetized with a mixture of xylazine and ketamine as described above and a laparotomy was performed along the incision made during PRV-tracing to re-expose the abdominal surface of the diaphragm. Bipolar hook electrodes (PFA coated tungsten wire with exposed tips, A-M Systems, Cat # 796500) were then placed into the medial costal region of the left and right hemidiaphragm as previously described (Lane et al. 2012). Activity was recorded during spontaneous breathing and baseline was counted as a minimum of 10 minutes of stable activity. Once baseline was acquired, a nose cone was placed over the animals’ nose, which allowed the administration of hypoxic gas (10% O2, flow rate 2L/min) as means of a respiratory challenge for 5 minutes. The dEMG signals were amplified (1,000x) and band pass filtered (0.3-10KHz) using differential A/C amplifier (Model 1700, A-M Systems) and digitized (Power 1401, Cambridge Electronic
Design).

4.3.8 Immunohistochemistry

At the end of terminal recordings, all animals were intracardially perfused with saline (0.9% NaCl in water) and paraformaldehyde (4% w/v in 0.1M phosphate buffered saline (PBS), pH 7.4). The spinal cords were then removed and stored in paraformaldehyde overnight. Longitudinal and transverse tissue sections (20um, on slide) spanning caudal-most part of brainstem to beginning of thoracic spinal cord (T1-2) were cryoprotected (15%, then 30% sucrose, overnight), and sectioned using a cryostat.

Sections were rehydrated for 15 minutes in PBS, blocked against endogenous peroxidase activity (30% methanol, 0.6% hydrogen peroxide in 0.1M PBS, incubated for 1 hour), and blocked against non-specific protein labeling (10% serum in 0.1M PBS with 0.02% Triton-X, incubated for 1 hour), prior to administration of primary antibodies in blocking solution. Primary antibodies (summarized in Table 1) were left on the tissue overnight at room temperature. The following day, tissue was washed in PBS (0.1M, 3 x 5 minutes) and incubated in blocking solution with secondary antibodies (summarized in Table 1). Immunolabeled sections were then washed in PBS (0.1M, 3 x 5 minutes), allowed to dry, and coverslipped with fluorescence mounting medium (Dako). Sections were examined using a Zeiss AxioImager microscope with Apotome 2, attached to a Dell PC. Photographs were taken with a digital camera (Zeiss Axiocam MRm).
Table 4.1: Primary and Secondary Antibodies

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<th>Concentration</th>
<th>Vendor</th>
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<tbody>
<tr>
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<td>PRV 1:10,000</td>
<td>Courtesy of Lynn</td>
</tr>
<tr>
<td>Rabbit</td>
<td>NeuN 1:500</td>
<td>Abcam</td>
</tr>
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<td>Mouse</td>
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<tr>
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<tr>
<td>Guinea Pig</td>
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<table>
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<td>Molecular Probes</td>
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</tr>
<tr>
<td>Anti-guinea pig DyLight 649 1:200</td>
<td>Jackson</td>
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4.3.9 Data Analyses

Anatomical and electrophysiological results were quantified using Microsoft Office Excel 2016, Spike 2 (version 8, Cambridge Electronic Design, UK) and OriginLab9 software (Northampton, Massachusetts). Mean values are shown as ± standard deviation. Statistical analyses were performed using SPSS (IBM SPSS Statistics 23). Comparison between injured and naïve groups was made using ANOVA and Student t-test (IBM SPSS Statistics 23). All analyses of electrophysiological data were blinded.

4.4 Results

4.4.1 Animal Survival after Spinal Cord Injury

The spinal cord injury (SCI) induced for this study is one of high cervical (C3/4), moderate-severe injuries that results in both white and gray matter damage around the phrenic motor pool (C3-6). All injured animals that experienced respiratory arrest were immediately
intubated and mechanically ventilated for one hour. Although a total of 35 animals received a lateralized contusion injury with the impact force preset to 200 kD (actual force: 264 ± 68kD), 7/35 of the animals either did not wean from the ventilator and died shortly after SCI (within 1 hour of SCI) or did wean but did not recover post-injury (died within one week after SCI). The transplantation paradigm used in this study is sub-acute (within one week after injury), increasing the risk of morbidity and mortality in a population of injured animals. An additional 7 out of total 35 animals did not recover after transplantation surgery and either died overnight or were euthanized due to post-surgical complications.
Figure 4.1: Aggregation of ESC-V2a, ESC-pMN, and NPCs. (A-C) NPCs isolated from E13.5 rat spinal cords were stored frozen and thawed one day prior to aggregation (Scale bars = 200 µm). (D-F) Chx10-Puro ESCs expressing TdTomato and (G) Olig2-Puro ESCs expressing eGFP were induced using an appropriate 2-i/4+ protocol followed by 24 hours of puromycin selection (Scale bars = 200 µm). (H) Schematic showing aggregation protocol. As appropriate, populations were mixed at a 1:1 ratio and seeded into an Aggrewell plate (I). After 2 days of culture, spherical aggregates form in the dish (J), which are then resuspended in fresh media and washed in HBSS prior to transplantation (K) (Scale bars = 200 µm). (L) Cells within the aggregate are randomly distributed and form interpenetrating networks of neurites (Scale bar = 100 µm).
4.4.2 Characterization of Aggregates for Transplantation

Because of the hostile environment of the injury site, transplantation of post-mitotic neurons after SCI has historically resulted in poor cell survival. NPCs or ESC-pMNs were co-transplanted as aggregates in order to provide physical and trophic support to the ESC-V2as, as well as to provide additional cellular diversity for improved circuit formation. Five different groups were evaluated for their efficacy: (1) ESC-V2as with NPCs (V2a/NPC) (n=6), (2) ESC-V2as with ESC-pMNs (V2a/pMN) (n=3), (3) ESC-V2as (n=2), (4) NPCs (n=2), and (5) ESC-pMNs (n=6), including a vehicle-only control (HBSS, n=2). Briefly, NPCs and ESC-derived populations were thawed or differentiated and selected as previously described (Iyer et al. 2016a; McCreedy et al. 2012), then cultured for 2 days in commercially available Aggrewell plates to form stable aggregates that demonstrate interpenetrating networks of neurites (Figure 4.1). Although mixed groups were seeded at a 1:1 ratio, proliferation in NPC and pMN populations during the aggregation timeframe diluted the V2a population to 36.55 ± 1.63% and 43.85 ± 6.62% as a proportion of cells in the neuroaggregates respectively. The size differential between aggregates can be ascribed to this proliferation; groups containing NPCs or pMNs averaged approximately 120 µm in diameter, but ESC-V2a aggregates were slightly smaller (104.9 ± 14.63 µm) (Table 4.2). In order to determine cell viability, aggregates were enzymatically dissociated by prolonged incubation in Accutase; despite shear caused by trituration, all groups contained greater than 90% live cells (Table 4.2).
Table 4.2: Quantification of cell viability, population distributions, and aggregate sizes. Values presented as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>% Cell Viability</th>
<th>% V2a</th>
<th>Diameter (µm)</th>
<th># Aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC</td>
<td>94.61 ± 0.87</td>
<td>0</td>
<td>125.55 ± 16.05</td>
<td>512</td>
</tr>
<tr>
<td>ESC-V2a</td>
<td>91.82 ± 3.87</td>
<td>100</td>
<td>104.9 ± 14.63</td>
<td>388</td>
</tr>
<tr>
<td>ESC-pMN</td>
<td>94.77 ± 1.32</td>
<td>0</td>
<td>120.11 ± 20.67</td>
<td>443</td>
</tr>
<tr>
<td>V2a/NPC</td>
<td>96.16 ± 0.81</td>
<td>36.55 ± 1.63</td>
<td>124.35 ± 20.09</td>
<td>498</td>
</tr>
<tr>
<td>V2a/pMN</td>
<td>93.79 ± 1.72</td>
<td>43.85 ± 6.62</td>
<td>122.11 ± 18.38</td>
<td>352</td>
</tr>
</tbody>
</table>

Figure 4.2: Survival of transplants 1 month post-injury. Fluorescence from (A) NPCs (B) ESC-V2as, (C) ESC-pMNs, (D) V2a/NPCs and (E)V2a/pMNs apparent within the spinal cord lesion 1 month post-injury. Proliferation of GFP$^+$ progenitor populations likely contributes to larger transplant area than groups containing Tdtomato$^+$ ESC-V2as (Scale bars = 250 µm).
4.4.3 ESC-V2as Survive and Migrate 1 month Post-Transplantation in a Sub-Acute Cervical Contusion Model of SCI

The first objective of this study was to determine the feasibility of transplanting ESC-V2as into a lateralized C3/C4 contusion model. Aggregate transplantation was delayed 1 week after injury to allow for glial scar stabilization and for improved cell survival. Immunofluorescence analysis was conducted 1 month post-injury. TdTomato\(^+\) ESC-V2as were observed in all relevant groups (Figure 4.2), though survival of ESC-V2a INs transplanted alone was low compared to the V2a/NPC and V2a/pMN groups (Figure 4.2). In mixed transplant groups, the area occupied by progenitor populations was demonstrably greater than that of ESC-V2as (Figure 4.2A-E), which can be attributed to the proliferation of progenitor and glial cells. However, attrition was observed in groups containing mouse ESC-derived populations, likely caused by macrophage infiltration evidenced by fluorescent debris throughout the lesion and the presence of ED1\(^+\) cell bodies (Figure 4.3). The lower quantity of ED1 staining in NPC groups (Figure 4.3A-C) suggests an immune response caused by species mismatch despite continuous immunosuppression with CSA.
Figure 4.3: Macrophage infiltration into SCI lesion and graft. Representative images of ED1\(^+\) macrophages (red) about the spinal cord lesion on ipsilateral and contralateral sides in (A-C) NPC-treated and (D-F) ESC-pMN-treated groups. (Scale bars = 250 µm).

Although cell survival was compromised, migration of transplanted cells across the host-graft interface (as defined by GFAP staining) was observed in V2a/NPC, V2a/pMN, NPC, and ESC-pMN groups (Figure 4.4). GFP\(^+\) cell bodies from groups containing NPCs were able to migrate up to 2mm from the injury (Figure 4.4A-C); though they did not present with the classic molecular markers, these cells were morphologically similar to glia. ESC-V2as from V2a/NPC or V2a/pMN transplants were able to extend neurites up to 1mm towards the central canal or rostral of the injury (Figure 4.4C, inset), but ESC-V2as transplanted alone remained within the lesion (data not shown). GFP\(^+\) and TdTomato\(^+\) neurites from a V2a/pMN transplant were also observed entering the ventral horn (Figure 4.4D), may signify a potential for downstream muscle innervation. These data suggest that not only is the transplantation of post-mitotic ESC-V2as
feasible, but that these cells are able to bypass the lesion and integrate with host tissue if co-transplanted with supportive progenitor populations.

Figure 4.4: Migration of ESC-V2a neurites and progenitor-derived glia and neurons beyond glial scar. Representative images from longitudinal sections of (A) GFAP+ astrocytes (red), (B) GFP+ NPCs (green) and TdTomato+ ESC-V2as (white) migrating from the SCI lesion. (C) NPCs were able to migrate 2 mm beyond the glial scar (Scale bars = 1 mm); (inset) TdTomato+ ESC-V2a neurites were observed 1 mm from the injury (D) V2a/pMNs transplants were able to extend neurites from the graft into the ventral horn (Scale bars = 100 µm).
Figure 4.5: Maturation of transplanted cells. (A) TdTomato\(^+\) ESC-V2as were negative for the proliferation marker Ki67 (blue), but GFP\(^+\) NPCs demonstrate co-localization with Ki67. Fluorescent analysis of (B) ESC-V2a, (C) V2a/pMN, and (D) V2a/NPC for the mature neuronal marker NeuN (blue) and astrocyte marker GFAP (white). ESC-V2a transplants show contamination with unknown GFP\(^+\) populations (Scale bars = 100 µm).
4.4.4 Maturation and Differentiation of ESC-V2a INs and Co-transplanted Populations

Differentiation of ESC-pMNs and NPCs was investigated using immunohistochemistry to determine what terminal cell types were interacting with and supporting transplanted ESC-V2as. NPCs stained positive for the proliferation marker Ki67, but TdTomato+ ESC-V2as were negative, confirming their post-mitotic identity as previously reported *in vitro* (Iyer et al. 2016a) (Figure 4.5A). Cross-contamination of ESC-V2a transplants with GFP+ cells was observed, possibly due to an error during transplantation procedures. There was robust co-localization of GFP+ ESC-pMNs and NPCs with GFAP (astrocytes) within relevant transplant groups (Figure 4.5C, D). NeuN was expressed in V2a/NPC transplants, but was negligible in all other conditions. All transplanted populations also failed to stain for vesicular glutamate transporter 2 (Vglut2), a characteristic marker for excitatory neurons including V2a INs, or choline acetyltransferase (ChAT), which marks cholinergic MNs (data not shown). Inconclusive staining in these populations could indicate neuronal immaturity or distress, or could be a result of other pathological changes (Gusel'nikova and Korzhevskiy 2015).
4.4.5 Spontaneous Functional Activity in Transplants Containing ESC-V2as

Respiratory activity following cervical hemi-contusion is characterized by reduced baseline activity on the side ipsilateral to the injury compared to the contralateral side, as well as a blunted response to respiratory challenge compared to uninjured controls (Hoh et al. 2013). Bilateral terminal recordings of diaphragm electromyography (diaEMG) were analyzed to detect...
whether transplant recipients exhibited improved activity during spontaneous breathing or hypoxic challenge 1 month post-injury compared to injured, vehicle control groups (Figure 4.6). Preliminary data did not demonstrate statistically significant differences between groups (Figure 4.6A,B), but importantly, the transplantation of ESC-derived V2a INs and pMNs did not impair spontaneous recovery. The upward trend in activity between NPC and V2a/NPC groups suggests that the inclusion of ESC-V2a INs is responsible for the enhanced function. Transynaptic retrograde tracing with PRV was performed in order to determine if ESC-V2as within V2a/NPC treated animals integrated into phrenic circuits; initial findings suggest that they do not, which leaves the mechanism of action as yet unknown.

4.5 Discussion

4.5.1 Respiratory Model of SCI to Investigate ESC-Derived Interneuron-Mediated Remodeling

This study demonstrates for the first time the successful transplantation, survival, and migration of ESC-derived V2a INs after SCI. Although previous work using pluripotent stem cells, NPCs or fetal spinal cord (FSC) contained neuronal progenitors capable of differentiating into mature neuronal populations (Kobayashi et al. 2012; Lu et al. 2014; Nutt et al. 2013; Ogawa 2002; White et al. 2010), direct investigation into the role of a single IN population on functional recovery has until recently been infeasible. The unilateral C3/C4 cervical contusion injury used here is of moderate to high severity, resulting in the disruption of both white and gray matter tissues. This compromises phrenic motor pathways responsible for innervation to the diaphragm, resulting in respiratory deficits common in cases of human cervical SCI (Lane et al. 2008a; Lane et al. 2009). However, spontaneous functional recovery is possible in these rodent models, and
has been attributed in part to compensatory mechanisms mediated by local interneuron populations (Lane 2011; Lane et al. 2009; Lane et al. 2008b). V2a INs, which have known roles in the coordination of respiratory activity (Crone et al. 2012; Romer et al. 2016), were therefore a candidate population for transplantation to enhance recovery.

Because of issues with cell viability often associated with transplanting post-mitotic neurons, we co-transplanted ESC-V2as with NPCs or ESC-pMNs. Culturing them as neuroaggregates in vitro just prior to injection into the spinal cord allowed the formation of stable networks that could buffer the ESC-V2as against the toxicity of the SCI environment. Co-transplantation with progenitors also provided populations with which to form functional networks. NPCs have been shown to differentiate into a wide variety of cell types in vivo, including excitatory and inhibitory neurons, astrocytes, and oligodendrocytes (Tetzlaff et al. 2011). pMNs are restricted to motor neuron, astrocyte, and oligodendrocyte lineages (Novitch et al. 2001; Takebayashi et al. 2002), which offer fewer opportunities for complex network formation, but could provide greater insight into the specific role of ESC-V2a INs in exclusively driving any functional recovery.

4.5.2 ESC-V2as are Viable Candidates for Cell Transplantation Investigations

Previous studies confirmed that transplanted Olig2-Puro ESC-derived pMNs do not have tumorigenic properties despite their derivation from a pluripotent source (McCreedy et al. 2014b; Wilems et al. 2015); here we verified that Chx10-Puro ESC-derived V2a INs also do not generate tumor-like growths (Johnson et al. 2010b) or stain for the proliferation marker Ki67. Importantly, while ESC-V2a transplantation did not generate significant behavioral improvements, neither were they an impediment to spontaneous recovery (Johnson et al. 2010b).
Immunofluorescence analysis of ESC-pMN or ESC-V2as suggest that they do not attain maturity, which belied observations of neurite processes exiting the graft and work showing neuronal differentiation of ESC-pMN in vivo two weeks post-transplantation in a sub-acute dorsal hemisection model of spinal cord injury (McCreedy et al. 2014b; Wilems et al. 2015). Though some NPCs cells from V2a/NPC grafts were NeuN+, inconclusive staining for neurotransmitter transporters reinforces the possibility that the transplants remain immature 1 month post-transplantation.

Despite immunosuppression, the persistence and prevalence of inflammatory macrophages distributed both ipsilateral and contralateral to the lesion in groups containing mouse ESC-derived populations may contribute to the death of maturing neurons and oligodendrocytes (David et al. 2012). NPC groups showed lower quantities of ED1 staining, which may have allowed maturation to occur at a faster rate. Previous work with ESC-pMN has involved transplantation in growth-permissive fibrin scaffolds coupled with sustained release of neurotrophins (McCreedy et al. 2014b; Wilems et al. 2015); this comparatively pro-regenerative environment likely enhanced the viability of pMN-derived motor neurons and glia. A similar approach could be taken here, using the platform described in Chapter 3 to optimize combinatorial strategies prior to transplantation. Alternatively, transplanting cells the level of injury may be more permissive to engraftment and integration into host networks, promoting the formation of bridge circuits around the lesion instead of through it. A recent study examined the effect of human stem cell derived inhibitory interneuron precursors transplanted into the lumbar spinal cord after a moderate thoracic contusion model of SCI in mice. By augmenting the GABAergic tone in the lumbar microcircuitry, they found improved bladder function and
mitigation of pain-related symptoms 6 months after transplantation (Fandel et al. 2016).

Longer studies would clarify whether ESC-V2as and ESC-pMNs mature with time in this model, and whether these mature neurons have an impact on functional recovery. While the mixed groups used in this study were seeded at a 1:1 ratio, changing this proportion or the addition of other IN populations, including ESC-V3 INs (Xu et al. 2015), could alter electrophysiological properties in the transplant. Unpublished work from the Pfaff lab examined the effect of titrating cell sorted ESC-derived inhibitory V1 INs into populations of ESC-derived excitatory V3 INs; counter to intuition, increasing the number of inhibitory cells in the population resulted in proportional increases in the frequency of action potential firing in V3 INs. The addition of V1 INs into ESC-derived MNs did not change firing frequency, but did cause the formation of discrete MN microcircuits that fired asynchronously from one another, compared to synchronous firing in MN cultures without V1 INs. These studies suggest that ESC-derived IN populations can retain intrinsic rhythm generation properties in vitro, and that changing the culture composition can have a dramatic impact on network activity (Pfaff 2016).

4.6 Conclusions

In this preliminary study we demonstrate the feasibility of transplanting highly enriched populations of ESC-V2as after SCI, even in the absence of a growth-permissive environment. Survival and integration were improved by the co-delivery of precursor cells, though maturation in all of these populations remained minimal 1 month post-transplantation. We now need to determine how best to enhance the survival, maturation, and integration of these cell types to improve functional recovery. As the development of human stem cell-derived V2a IN
populations comes into fruition, these studies become more important for eventual clinical translation.
Chapter 5

Summary and Future Directions

5.1 Summary of Findings

The work of this dissertation has advanced tools and models to study the role of V2a interneurons (INs) in regeneration after spinal cord injury (SCI). Although V2a INs are one of the cardinal ventral IN populations, the limitations of \textit{in vivo} models have previously made such investigations infeasible. Here, we turn to embryonic stem cells (ESCs) as a renewable, unlimited source of V2a INs for \textit{in vitro} study and transplantation. Previous work in the lab optimized the differentiation of V2a INs from ESCs (Brown \textit{et al.} 2014b), but the heterogeneity of cultures in combination with the presence of proliferative, undifferentiated ESCs prevented these populations from being used for translational studies. To overcome this hurdle, we applied a genetic strategy to enrich V2a INs from this mixed population. In Chapter 2 we reported the generation of a selectable Chx10-Puro ESC line that expresses the puromycin resistance gene, puromycin-N-acetyltransferase under the native gene regulatory elements of Chx10, the defining transcription factor for V2a INs. After induction and the application of puromycin, more than 80% of surviving cells were Chx10$^+$. These cultures stain for the mature, post-mitotic markers of V2a INs, and develop electrophysiological activity characteristic of normal glutamatergic neurons. Furthermore, in a simple adherent co-culture of ESC-derived V2a INs (ESC-V2as) and
ESC-derived progenitor motor neurons (ESC-pMNs), functional synapses were observed, indicating a potential for more complex network formation. Although the ESC-V2as did not have identical molecular or electrophysiological properties as populations that develop in the spinal cord, the sufficient similarities allowed us to consider using these cells for modeling spinal microcircuits and for transplantation after spinal cord injury.

In Chapter 3, we used these ESC-V2a and ESC-pMN populations as part of an *in vitro* aggregate platform similar to the dorsal root ganglion (DRG) assay to investigate whether co-culture and/or various neurotrophins might have a differential, dose-dependent effect on the regeneration and maturation of these INs. In varying concentrations of glial-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and platelet-derived neurotrophic factor (PDGF), we found that ESC-V2as did not demonstrate an appreciable response. However, when co-cultured with ESC-pMNs, ESC-V2as experienced improved neurite extension, long-term cell survival and maturation, and rhythmic electrophysiological activity. These effects could be enhanced when growth factor treatments were optimized; GDNF and PDGF in particular were found to have significant effect on co-cultured ESC-V2as. Preliminary studies suggest that, because these growth factors are bound exclusively by ESC-pMNs in these neuroaggregates, that the maturation and proliferation of motor neuron (MN) and oligodendrocyte populations had an indirect, positive effect on ESC-V2a response.

Finally, we wanted to determine if the transplantation of these ESC-V2as into an SCI model was feasible, since the transplantation of post-mitotic neurons has typically resulted in poor viability. Using the neuroaggregate format developed in Chapter 3, in Chapter 4 we
transplanted neural progenitor cells (NPCs) or ESC-pMN enriched with this ESC-V2as into a sub-acute C3/C4 lateral contusion model of SCI in rats. This model was of particularly high severity, resulting in significant white and gray matter loss as well as phrenic motor pool deficits resulting in impaired breathing. After 1 month post-injury, the presence of ESC-V2as was detected in groups where ESC-V2as were transplanted alone as well as those co-transplanted with progenitors; the addition of progenitors enhanced the degree of ESC-V2a survival, as well as the overall volume of the graft. Both progenitor populations and ESC-V2as were capable of migrating up to 2 mm from the injury site on the rostrocaudal axis; ESC-pMN and ESC-V2as were also observed exiting the spinal cord through the ventral roots. However, the infiltration of macrophages into transplants containing mouse ESC-derived populations resulted in attrition, possibly due to species mismatch despite continuous immunosuppression. The presence of neuronal maturity markers, even after 1 month, was also minimal. Even so, an assessment of breathing function by diaphragm electromyography showed that V2a/NPC transplantation significantly improved functional recovery compared to NPCs alone, ESC-pMN, V2a/pMN, or vehicle-control groups. While retrograde tracing of phrenic motor pools failed to show connectivity between phrenic MNs and ESC-V2as, the data validates the potential of these populations as part of transplantation strategies and suggests a probable role in functional recovery.

### 5.2 Recommendations for Future Directions

Given the scarcity of IN-specific research across traumatic and degenerative
neurophysiological conditions, the introduction of the tools developed herein to pre-existing research paradigms has the potential to generate novel clinical insights. In this section I describe possible applications for ESC-derived V2a INs in ongoing work in the Sakiyama-Elbert lab, with collaborators, and beyond.

5.2.1 Combinatorial Therapies

SCI is characterized by multiple systemic and pathological obstacles that are not easily overcome by any single treatment—some combination of physical therapy, electrical stimulation, biomaterials scaffolding, cell replacement, and pharmacological intervention may be required. The Sakiyama-Elbert lab in particular focuses on the latter three strategies. While the lab has historically concentrated on controlled drug delivery within fibrin matrices, future work could optimize those scaffolds for the efficacious delivery of ESC-derived ventral INs to the injury site. As evidenced in this thesis, the transplantation of V2a INs—a post-mitotic neuronal population—poses unique challenges.

Despite the many benefits of cell transplantation, the hostile, toxic environment of the injury site is a significant detriment to cell survival. The aggregate method described in Chapters 3 and 4 was developed in part to improve the post-transplantation survival of the selected populations, which require dissociation from EBs in order to eliminate all remnant stem cells, but the use of a biomaterials scaffold could further diminish attrition caused by the lesion environment. A wide variety of biomaterial scaffolds have been investigated as transplantation vehicles and are typically chosen to accomplish one or more goals, including the protection of
transplanted populations, providing a structural bridge between injured areas, promoting axonal extension and cell migration, and sustained delivery of pharmacological therapies (Raspa et al. 2016). Protein-based biomaterials tend to have superior biocompatibility and an abundance of cell adhesion sites, which are critically important because they assist in cell survival, migration, proliferation, and differentiation. Compared to natural materials, synthetic scaffolds offer more customizable attributes and greater control over mechanical properties, but their use can be limited by enhanced inflammatory and immune responses, few cell adhesion sites, and poor engraftment into the host tissue (Shrestha et al. 2014; Straley et al. 2010). Balancing the positive and negative properties of a biomaterial is important when tailoring scaffold design for specific cell and tissue types.

Systemic delivery, scaffold-based delivery, and micro- and nano-particle based delivery systems are the three main strategies to deliver pharmacological factors after SCI (Elliott Donaghue et al. 2016; Lee et al. 2010; Tyler et al. 2013; Wang et al. 2011; Willerth and Sakiyama-Elbert 2007; Zhao et al. 2013). Neurotrophins and growth factors are often investigated, as well as anti-inhibitory molecules, anti-convulsants, immunotherapeutic molecules, hormones, and antibody treatments, many of which are in current use or in clinical trials (Kabu et al. 2015; Mohtaram et al. 2013; Silva et al. 2014). Because more controllable drug delivery systems are not necessarily compatible with cells—harsh synthesis methods can result cell toxicity or a heightened immune response—combinatorial systems that are able to co-deliver drugs and stem cell-derived populations are still in the early stages of development. The Sakiyama-Elbert lab has done significant work demonstrating the effectiveness of using fibrin scaffolds to co-deliver pharmacological factors with ESC-derived NSCs or pMNs (Johnson et al.
though feasible, these approaches still require refinement to improve overall efficacy (Karimi-Abdolrezaee et al. 2010; Wilems et al. 2015).

An as yet poorly explored facet of combinatorial therapy is the precise ratio of spinal cell populations delivered to the injury site. Typically, neural stem cells are transplanted with the expectation that they will differentiate into both glia and neurons in vivo post-transplantation, but these processes are often uncontrolled and do not necessarily or intuitively differentiate into the populations required for functional recovery. The generation of multiple selectable cell lines for spinal cell populations—including those for the V2a INs described here, V3 INs, V0 INs, motor neurons (MNs), pMNs, and astrocytes—has permitted the Sakiyama-Elbert lab to begin testing for ideal combinations both in vitro and in vivo (Iyer et al. 2016a; McCreedy et al. 2014a; McCreedy et al. 2012; Xu et al. 2015). In this thesis, simple 1:1 mixtures of pMNs, V2a INs and NPCs were used for co-culture in Chapters 2, 3 and 4, but future work could optimize these for different types of injuries at different levels of the spinal cord. Indeed, unpublished work from the Pfaff lab is exploring this very concept using lineage traced ESCs instead of the selectable lines developed by our lab. Using cell sorting to isolate a combination of V1 INs, V3 INs and MNs from ESC-derived cultures, their work demonstrates that increasing the proportion of inhibitory V1 INs in relation to excitatory V3 INs and MNs results in increased firing frequency and the development of asynchronous, regional activity in these populations respectively. With the development of more precise in vitro and in vivo differentiation strategies, the reliance on transgenic approaches could be reduced or eliminated in time.
5.2.2 SCI Studies in Lineage Traced Mice

No markers have been determined to identify mature ventral IN populations, thus typical immunohistochemistry and tract tracing methods are incapable of identifying the specific INs involved in local rewiring events observed after SCI in vivo. Transgenic lineage tracing mice provide the unique opportunity to study how endogenous IN populations behave after trauma. Although physical sprouting of ventral IN populations rostral to the lesion site would be interesting and meaningful to observe—and in line with 2008 findings by Courtine et. al.— it is more likely that, given their interdependent roles in rhythm generation, electrophysiological changes associated with compensatory activity would be more prominent (Arber 2012; Courtine et al. 2009; Courtine et al. 2008; Jankowska 2001). The Harris-Warrick lab has done some work in this area for V2a INs in neonatal mice, demonstrating pronounced changes in serotonin sensitivity exclusive to these populations (Husch et al. 2015; Husch et al. 2012). More work ought to be done in adult animals, where spinal networks are less plastic. As discussed in Chapter 1, while the literature suggests that ventral INs are candidate populations to target following SCI, there is little direct evidence to prove that they alone are responsible for functional remodeling. Although groundwork in these models is needed, applying combinatorial therapies that include ESC-derived IN populations to lineage-traced animals is of great interest. Regenerating axons and/or transplanted cells need to activate intact CPG circuits in order to induce coordinated locomotion. Using molecular and histological approaches to detect sprouting and synapse formation between endogenous and grafted populations would motivate the development of more complex animal models that incorporate chemogenetic or optogenetic
technologies, as well as \textit{in vitro} assays to better characterize and promote specific functional connections. These ideas are discussed in the sections that follow.

\subsection*{5.2.3 Network Physiology \textit{In Vitro} and \textit{In Vivo}}

Testing the efficacy of transplantation in animal models often relies on a combination of behavioral and electrophysiological assessments to measure gross sensorimotor recovery coupled with molecular and histological assessments to quantify neuronal outgrowth, differentiation, and synapse formation (Abematsu \textit{et al.} 2010; Bonner \textit{et al.} 2011; Fujimoto \textit{et al.} 2012; Johnson \textit{et al.} 2010c). However, a deep understanding of the developing network properties at the cellular level—between neurons within the graft, and between the graft and host—are missing from current approaches because of the challenges making these measurements \textit{in vivo}. These types of analyses are critical if we are to design SCI treatments that improve the transmission of activity through the transplant and across the lesion. \textit{In vitro} modeling of these interactions may prove valuable towards that end; transcription factor-driven selectable ESC lines can be used modularly to generate complex cultures that remain extremely well defined. An accurate \textit{in vitro} model of SCI or central pattern generator networks would allow for screening of drugs, trophic factors, or electrical stimulations to aid functional interactions between regenerating and intact circuits.

In collaboration with Dr. Dennis Barbour, we are developing an \textit{in vitro} microelectrode array (MEA)-based platform to generate high throughput paired recordings between our enriched ESC-derived INs and primary cell populations, working to model transplant-transplant and host-transplant interactions. Using such platforms, we can monitor synaptic connectivity within these populations over distances equivalent to multiple spinal segments and refine manipulations with
pharmacological factors. Multi-component systems that include fabricated microchamber devices may be formed to physically separate cell populations and direct their growth (Lu et al. 2012c). Although simplified networks formed in vitro do not reflect the inordinate complexity of the central nervous system, they can inform our understanding of intrinsic cellular properties oft obscured by environmental concerns that could offer insight into critical targets for repair.

### 5.2.4 Chemogenetics and Optogenetics-Mediated Control of ESC-Derived Transplants

Chemogenetic and optogenetic tools are becoming ubiquitous, powerful techniques that enable neuroscientists to gain mechanistic insight into neural networks in vivo in a reversible, rapid, and minimally invasive manner; there are many potential uses of these technologies to treat spinal cord injury (Ahmad et al. 2015). As discussed in Chapter 1, chemogenetic or optogenetic modifications to transplantable stem cell-derived populations would enable us to evaluate how transplanted cells contribute to behavioral gains without relying on ex vivo electrophysiology preps, re-transection, or excitotoxic ablation. Because the engineered proteins are reversibly activated or inactivated using clozapine-N-oxide or a light stimulus, we could observe the time course of transplant maturation frequently, more logically design time-points to do histological assessments, investigate the transmission of activity through graft, and actively stimulate transplanted cells to remodel (Creasey et al. 2004; Karadimas et al. 2016; Montgomery et al. 2016). When used modularly with selected ESC-derived IN populations, such assays could be used to determine the contributions of genetically unique IN populations both in vivo and in vitro to functional network properties; the application of optogenetic and chemogenetic tools to
the MEA platform previously described would add an additional facet of control to a relatively simple network.

5.2.5 Screens for Cell Fate Determination

The complexity of the adult spinal cord is a significant barrier to the development of therapeutics that target specific, regenerative cell populations. While prime populations—motor neurons, glia, sensory neurons, etc.—and their subpopulations are generally well defined, there are many IN populations that are ignored, poorly characterized, or remain unidentified because no unique markers have as yet been discovered. Developmental biologists currently rely on time and resource-consuming in situ hybridization screens and/or genetic ablation studies to find “novel” populations and their defining transcriptional markers. Our selectable ESC-derived IN populations could mitigate the need to sacrifice animals for screening purposes while enabling us to use new technologies to map time-dependent genetic events responsible for diversification in specific cardinal interneuron populations.

In an ongoing collaboration with Dr. Rob Mitra of the Genetics Department at the Washington University School of Medicine, we have worked to develop tools that allow us to identify the key transcription factors, binding sites and key enhancer elements for regulation of gene expression during cell fate determination. The strategy involves the tamoxifen-inducible integration of markers that can be analyzed using next generation sequencing methods to find novel enhancer elements. The combination of the inducible screens and selectable ESC system is unique, since it allows for investigation of a single cell population at any point in its development and maturation. Alterations in the ESC induction protocol can be used to identify
changes in genetic programing that result in anatomical specificity or subtype diversification in ventral IN populations. This would allow us to further refine ESC induction protocols to obtain desirable populations for study or transplantation, but more importantly can be used to identify novel IN markers to parse spinal microcircuit architecture.

The benefits of these selectable ESC-derived populations for deconstructing genetic events are not necessarily limited to INs. The defining transcription factors used to drive selection appear in other tissues but serve different developmental roles. For example, the V2a marker Chx10 is also expressed in bipolar cells in the developing retina and is responsible for the expansion of retinal progenitor cells required for production of new retinal cells in adult animals (Dhomen et al., 2006; Rowan and Cepko, 2004). While we use ESC protocols optimized for spinal neuron culture (Brown et al., 2014), protocols exist to generate retinal tissue (Eiraku and Sasai, 2012) and might be used for similar cell fate determination studies.

5.2.6 V2a INs in Amyotrophic Lateral Sclerosis

Though amyotrophic lateral sclerosis (ALS) has historically been considered a disease selective for motor neurons and glia (Boillee et al. 2006), recent evidence suggests that V2a INs degenerate at late stages of the disease, and at early stages, may be involved in compensatory functions that enable continued respiratory function despite phrenic MN deficits (Romer et al. 2016). A significant body of research has been dedicated to studying ALS pathology in isolated MN cultures in vitro with the goal of identifying potential interventions to halt or reverse the course of degeneration (Boillee et al. 2006; Dimos et al. 2008; Nagai et al. 2007; Yang et al. 2008).
2013b); many of these types of studies could be translated to the V2a INs developed in this thesis. High-throughput screens could easily be applied to selected ESC-derived V2a INs, as could the introduction of SOD1 mutations to generate a genetic model of the disease. More interesting questions regarding disease pathology might also be explored: Do V2a INs follow the same trajectory of degeneration as MNs, but over a different time scale? Do diseased astrocytes and glia differentially affect the two populations, and if so, why? Are there possible interventions that could be used to halt IN degeneration at later stages, and thus retain compensatory function in vivo despite MN loss? As investigation of MNs in the context of ALS matures, there is now the potential for IN research to follow in its wake.

5.3 Concluding Remarks

Active engagement between translational researchers and basic scientists will be important for the field to move forward. High throughput gene expression methods, single-cell sequencing, proteomics, and similar techniques will allow for a more comprehensive investigation and comparison of cells generated during development and in the dish. The biology ought to inform both differentiation and therapeutic strategies. The complex interplay between transplanted cells, materials, pharmacological agents, and the in vivo environment will require combinatorial systems where the components complement, rather than detract from one another, as can so often be the case. Creative design is necessary to get these novel strategies from the bench top and into the clinic. I am so excited to see the future of this field; there is so much ahead of us.
Appendix A: MATLAB Code for Calcium Analysis

%%% The excel spreadsheet is set up such that the first column is the time step (the image number), the second column contains a sample of the background (a corner of the image where the background decay is visible). The rest of the columns are the individual ROIs. The code is set up to shuffle through each sheet of the excel book; the sheets are each named with the appropriate sample specifications. %}

[status,sheets] = xlsfinfo('CalciumImagingDataFormattxt.xlsx');

for k = 1:length(sheets);
    sheet_name = char(sheets(k));
    calcium_data = xlsread('CalciumImagingDataFormattxt.xlsx', sheet_name, 'A2:I752'); %change the range if there are more than 750 images or data points
    calcium_data = xlsread('CalciumImagingDataFormattxt.xlsx', 'A2:I752');

    time = calcium_data(:,1)*.25; %conversion to seconds
    background = calcium_data(:,1); %gets the background array
    calcium_trans = calcium_data(:,3:length(calcium_data(1,:)))); %gets the data array
    calcium_trans_adj = calcium_trans - repmat(background, 1, length(calcium_trans(1,:))); %subtracts the background from the data
    calcium_trans_corr = zeros(length(calcium_trans_adj(:,1)), length(calcium_trans_corr(1,:))); %creates a correction matrix to shift baselines

    %finds the baseline in each data vector and normalizes it to that vector
    for ii = 1:length(calcium_trans_adj(1,:))
        temp_calc = calcium_trans_adj(:,ii);
        calcium_trans_corr(:,ii) = temp_calc/min(temp_calc);
    end

    %plots the corrected calcium data in a figure
    figure(k)
    subplot(2,1,1)
    plot(time,calcium_trans)
    title(sheet_name);
    xlabel('Time (s)');
    ylabel('
\Delta F/F0')
    axis([0 185 0 inf])
    set(gca,'Box','off')
set(gca,'YTick',[])
set(gca,'YColor','w')
set(0,'defaultAxesFontName', '<Arial>')
set(0,'defaultTextFontName', '<Arial>')
ax.YAxis.Exponent = 4;
print('-djpeg','-r300',strcat(sheet_name,'.jpg'))
print('-depsc','-r300',strcat(sheet_name,'.eps'))
end
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Vita

EDUCATION
Johns Hopkins University, Baltimore, MD
B.S Biomedical Engineering; Minor: Mathematics  September 2007- May 2011

Washington University in Saint Louis, Saint Louis, MO
PhD Biomedical Engineering  September 2011-December 2016
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RESEARCH EXPERIENCE
Washington University in St. Louis, Biomedical Engineering 2011-present
Doctoral Student, Mentor: Shelly Sakiyama
Thesis: Developing High Purity Embryonic Stem Cell-Derived V2a Interneurons for In Vitro Investigation and Transplantation Following Spinal Cord Injury
• Thesis work funded by NIH NRSA F31 NS090760
• Developed a selectable mouse embryonic stem cell line to isolate Chx10⁺ V2a interneurons
• Developed an in vitro platform to characterize interactions between isolated populations of primary and transgenic embryonic stem cell-derived ventral interneuron populations to identify factors that assist in their survival, growth, and functional connectivity after spinal cord injury.
• Involved in additional collaboration work on projects involving peripheral nerve regeneration, cell replacement for spinal cord injury, and tools for stem cell fate determination

Ohio State University Spinal Cord Research Training Program  Spring 2014
• Trained to perform surgery, post-operative care, and behavioral and neuroanatomical assessments on various mouse models of spinal cord injury.

Washington University School of Medicine, Ophthalmology Spring 2012
Rotation Student, Mentor: Nathan Ravi
• Designed and implemented diffusive and mechanical testing of novel poly(acrylamide) vitreous substitutes and examined the effect of vitrectomy on the formation of free radicals on vitreous gels and substitutes.

Tsinghua University, Biomedical Engineering, Beijing, China  Summer 2010
Research Intern, Mentor: Jing Cheng
• Designed and tested microfluidics-based biochip platforms to evaluate sperm viability and motility for improved in vitro fertilization outcomes.

Johns Hopkins University, Materials Science and Engineering  2009-2010
Undergraduate Research Assistant, Mentor: Hai-Quan Mao
• Examined neural stem cell migration and differentiation on functionalized polycaprolactone nanofiber surfaces for use in neural guides for regeneration.

ImClone Systems, Cell and Tissue Engineering, New York, NY  
Summer 2009  
Research Intern  
• Evaluated the relative efficiencies of multiple mRNA regulatory sequences to optimize monoclonal antibody expression in human cells for use in cancer treatment.

Johns Hopkins Medical Institute, Neuroscience  
2008-2009  
Undergraduate Research Assistant, Mentor: Alex Kolodkin,  
• Examined Sema3A downstream players’ role in axon cone guidance in Drosophila using biomolecular and genetic techniques, specifically evaluating effects on microfibular structure and dendrite formation.

AWARDS  
NIH NRSA F31 Pre-Doctoral Fellowship 2014-2017  
Teaching Citation, Washington University in St. Louis 2015  
Travel Award, 16th International Symposium on Neural Regeneration 2015  
Travel Award, 2015 Biomedical Engineering Society Annual Meeting 2015  
Burroughs Wellcome Fund Travel Grant, 4th TERMIS World Congress 2015  
WU-CIRTL Practitioner Certificate 2014  
Deans List, Johns Hopkins University 2007-2011  
Bachelors’ Degree Awarded with Honors 2011  
Tau Beta Pi 2010-2011

PUBLICATIONS  


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**Professional Societies**

- Tau Beta Pi-Engineering Honors Society 2010-2011
- Biomedical Engineering Society 2007-Present
- Tissue Engineering and Regenerative Medicine International Society 2015-Present

**Teaching Experience**

**Bard College**

*Faculty of the Citizen Science Program* January 2017

- Developing curricula and independently teaching a Citizen Science class, an intensive core experience for first year students to give them a foundation in the conduct and content of science using problem-based learning, computational, and wet laboratory approaches.

**Washington University in St. Louis**

*Teaching Assistant*, Molecular and Cellular Biology for Engineers 2012, 2013
*Guest Lecturer*, Molecular and Cellular Biology for Engineers 2012, 2013
*Guest Lecturer*, Biotechnology Techniques for Engineers 2013
*AMGEN Mentorship* 2014, 2016

**Johns Hopkins University**

*Writing Consultant*, JHU Writing Center 2008-2011
*Lab Manager*, BME Models and Design 2009, 2010

**Coursework, The Teaching Center at Washington University in St. Louis**

- Teaching Assistant Orientation Summer 2012
- Teaching as Research (Scholarship of Teaching and Learning) Spring 2014

**STEM Pedagogies Workshops**

- Applying Cognitive Science to Improve Teaching
- Designing and Teaching an Introductory STEM Course
- Increasing Diversity and Improving Learning in STEM
- Incorporating Active Learning
- Inquiry-Based Laboratory Teaching: Philosophy and Implementation
• Designing Inquiry-Based Laboratory Materials
• Active Learning Strategy: Problem-Based Learning
• Designing Collaborative-Learning Materials
• Teaching Students to Critically Evaluate Scientific Literature

**Teaching Center Professional Development Workshops** 2012-2016
• Designing an Online Course
• Managing an Online Course
• Teaching with Lectures
• Teaching with Discussions
• Designing and Evaluating Writing Assignments
• Writing a Teaching Philosophy Statement

**COMMUNITY OUTREACH**
Young Scientists Program Continuing Mentorship 2012-2016
Each One Teach One College Bound Tutoring 2012-2015

**LEADERSHIP EXPERIENCE**
*Founding Chair*, BME Doctoral Council 2015-2016
Graduate Student Senate
  *Co-President* 2015-2016
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Graduate Research Symposium
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  *Committee Member* 2014-2015
Graduate School of Arts & Sciences Council
  *Executive Board* 2015-2016
  *Biomedical Engineering Representative* 2014-2016
Committee for BME Student Seminar Speaker Series 2015-2016
*Representative*, Professional Graduate Student Coordinating Committee 2015-2016
*GSS Representative*, Graduate Professional Council 2015-2016
Subcommittee for Student Affairs Strategic Plan 2015-2016
*Interviewer*, Johns Hopkins Admissions Alumni Volunteers 2011-Present
WUSTL Teaching Center Graduate Student Advisory Board 2012-2015
Biomedical Engineering Society Upperclassman Mentor 2009-2011
Randolph High School Forensics Team
  *President* 2006-2007
  *Publicity Manager* 2005-2006