THE DEVELOPMENT OF V3 INTERNEURONS IN THE MOUSE SPINAL CORD

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Abstract

V3 interneurons in the spinal cord are a group of excitatory commissural interneurons that play an important role in producing balanced and stable gaits in animals. In the developing mouse spinal cord, V3 interneurons arise from the ventral-most progenitor domain, p3. At embryonic day (E) 9.5, V3 enter the post-mitotic stage and express the transcription factor single-minded 1 (Sim1). Although Sim1 has long been used as a molecular marker to identify V3 neurons in the spinal cord, its function during V3 development is still largely unknown. To reveal its function, we have used several transgenic mouse models to knock out the expression of *Sim1* and to trace the fate of mutant V3 neurons during development. After leaving the progenitor domain, control V3 neurons migrate dorsally and laterally, settling at birth (P0) as clusters in Rexed's Lamina VIII (ventral), VII (intermediate), and IV-V (dorsal) regions. At early development (E12.5) axon projections in the caudal direction are ipsilateral, while axon projections in the rostral direction are contralateral. Through the remaining developmental days, ipsilateral projections diminish, while contralateral projections continue, eventually at birth projecting strongly in both the rostral and caudal directions. Although *Sim1* knockout does not alter the excitatory and commissural characteristics of V3 neurons, mutant V3s show a defective migration and axon projection pattern. In *Sim1* mutants, V3 neurons cluster with a less organized pattern. At P0, the dorsal subgroup cells are mostly absent, while intermediate cells are significantly increased, indicating *Sim1* mutant cells cannot correctly migrate. Furthermore, we found a drastic decrease in the number of ventral cells with both ascending and descending axon projections. A certain population of Non-Ventral V3 projections was also affected in *Sim1* knockout cells. This projection deficiency was seen at both E14.5 and P0 mice. *Sim1* plays a critical role in establishing the correct migration trajectory and axon projection profile of the V3 spinal interneuron.

List of Abbreviations Used

a	Ascending
acV3s	Ascending commissural V3s
aCINs	Ascending commissural projections
aV3s	Ascending V3s
ad	Ascending-descending
BMP	Bone Morphogenic Protein
CPG	Central pattern generator
CINs	Commissural interneurons
cV3s	Commissural V3s
comm	Commissureless
DCC	Deleted in Colorectal Cancer
d	Descending
dcV3s	Descending commissural V3s
dIINs	Descending ipsilateral interneurons
diV3s	Descending ipsilateral V3s
dV3s	Descending V3s
D	Dorsal
DsCAM	Down Syndrome Cell Adhesion Molecule
E	Embryonic
En-1	Engrailed-1
GDF7	Growth/differentiation factors 7
HTS	Hypothalamus-spinal cord
IaINs	Ia inhibitory interneurons
I	Intermediate
IN	Interneurons
LMC	Lateral motor columns
L	Lumbar
MB	Mammillary bodies
MTEG	Mammilotegmental tract
MTT	Mammilothalamic tract
MMC	Medial motor columns
MN	Motor neurons
PFA	Paraformaldehyde
PVN	Paraventricular nucleus
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Triton-X
P	Postnatal
p	Progenitor
RCs	Renshaw Cells
sCIN	Segmental commissural interneurons
Sema	Semaphorin
Sim1	Single-minded 1
$Sim1^{+/cre}$	Sim1 heterozygote
$Sim1^{tLacz/cre}$	Sim1 Knockout

Shh	Sonic Hedgehog
SON	Supraoptic nucleus
T	Thoracic
V	Ventral

To my Friends, my Family, the Lab, and the Manor

Chapter 1. Introduction

The neural networks that establish and maintain a balanced and coordinated gait are located within the ventral spinal cord (Deliagina et al., 1999; Jankowska, 2001; Kiehn, 2011). These neural networks are called central pattern generators (CPGs). The CPGs are initiated and modulated through descending inputs from higher brain regions (Deliagina et al., 1999; Grillner et al., 2005) as well as sensory afferents from the periphery (Rossignol et al., 2006; Windhorst, 2007). Recent studies surrounding the early development of the mouse spinal cord have brought profound breakthroughs in our understanding of the organization of CPGs, and the specific groups of neurons that comprise them. My project focuses on one of these genetically identified spinal neurons, *Sim1*$^{+}$ V3, with an aim to understand the developmental processes involved in integrating a population of spinal interneurons into a functional neural network.

1.1. The Development of ventral spinal neurons

Mature, functioning spinal neurons originate from progenitor cells located in the developing neural tube. Progenitor neurons are exposed to various gradients of morphogens secreted from either the dorsal roof plate and overlying epidermis (Bone Morphogenic Protein (BMP) and Wnt) or from the ventral floor plate and the notochord (Sonic Hedgehog (Shh)), depending on their location along the ventral-dorsal axis. These morphogens trigger a series of molecular events that

differentiate the progenitor cells into specific classes of neurons. The neurons that form the CPG networks predominately originate from the ventral neural tube and are induced by a gradient of Shh.

The concentration gradient of Shh directs the expression of certain transcription factors that will be expressed by mitotically active progenitor neurons (Ericson et al., 1997). These proteins, which are mainly homeodomain proteins, act as intermediary factors in the interpretation of the graded Shh levels, and can be divided into either Class I or Class II homeodomain proteins (Pierani et al., 1999; Briscoe et al., 1999; Briscoe et al., 2000). Class I proteins are repressed at a distinct Shh threshold (reviewed in Jessel, 2000) where as Class II proteins require a certain concentration of Shh in order to be expressed. The unique expression patterns of Class I and II transcription factors genetically define the different progenitor domains. The ventral boundary of a progenitor domain is established by the transition from repression to expression as Shh concentration decreases. The dorsal boundary of the progenitor domain is established by the transition from expression to repression as Shh concentration decreases.

Certain pairs of Class I and II proteins have cross-repressive characteristics that act to define the sharp boundaries between progenitor domains (Briscoe et al., 2000). For example, Nkx2.2 and 2.9 (class II) are induced by high levels of Shh, while Pax6 (Class I) is only expressed in more dorsal populations where the Shh concentration

is lower. This interaction creates a distinct progenitor domain, and establishes the border between the p3 and the pMN domain (Fig. 1).

Transcription factors downstream of the Class I and II proteins can lead neurons to exit the cell cycle to form mature neurons. For example, Nkx6.1 (pMN) directs the domain-restricted expression of the downstream factor MNR. MNR is expressed during the final division cycle of motor neuron progenitors and commits that neuron to the motor neuron identity (Tanabe et al., 1998).

The result of all this patterning is the establishment of 5 ventral progenitor domains (Jessel, 2000; Briscoe et al., 2000): motor neuron progenitors (pMN) and 4 classes of interneurons (p0-p3). These progenitor domains lead to the generation of subsequent post-mitotic populations: the motor neurons and V0-V3 interneurons (Fig. 1). They are genetically distinct, with key transcription factors playing integral roles in their developmental program. Recent studies have also shown that these genetically identified groups of spinal neurons play distinctive roles in control of locomotor activities. Next, I will briefly summarize the developmental process of the four groups of ventral interneurons in the spinal cord (See Fig. 2 for overview).

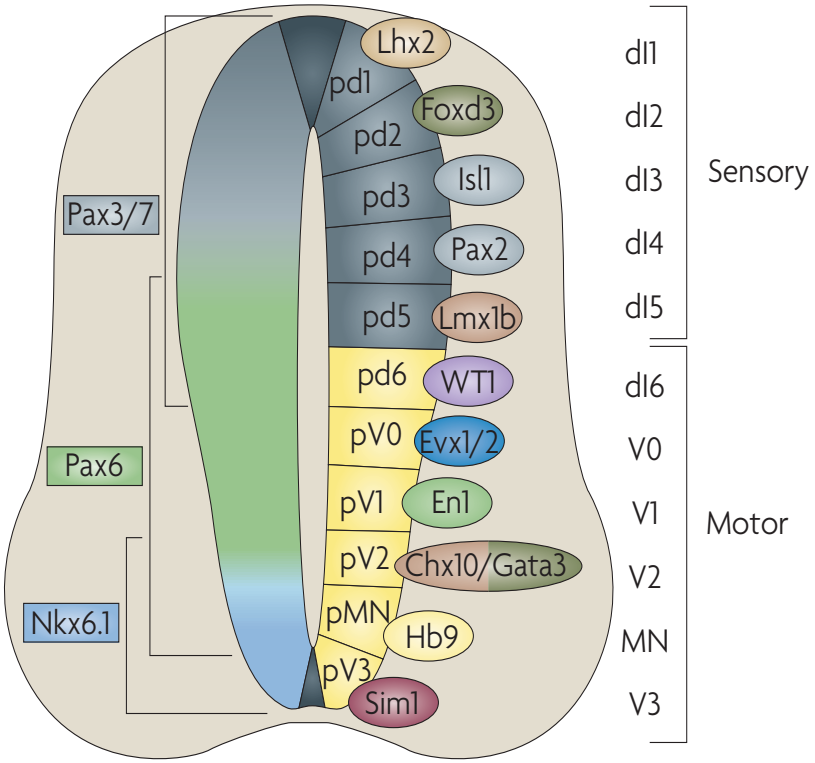


Figure 1. Early development of the spinal cord.

Schematic cross-section through the developing mouse spinal cord showing the patterning and specification of early spinal cord progenitors and their neuronal progeny. At E11, eleven early classes of postmitotic neurons are present in the embryonic spinal cord, with some of their identifying transcription factors listed. V3 interneurons arise from the ventral-most p3 progenitor domain, and express *Sim1*. Adapted from Goulding, 2009.

1.1.1. V0

The Evx1-expressing V0 interneuron population arises from the Dbx-1-expressing p0 progenitor domain, which is located just ventral to the sulcus limitans in the developing neural tube (Pierani et al., 1999, 2001). Most post-mitotic V0 interneurons migrate from their progenitor domain along a ventromedial pathway and congregate in the ventral horn within Lamina VIII (Moran-Rivard et al., 2001, Pierani et al., 2001). During this migration, these V0 neurons project commissural axons that form a fasciculate bundle within the ventral funiculus on the contralateral side. These axons travel rostrally 4 to 5 segments, and some of them synapse directly onto motor neurons (Moran-Rivard et al., 2001, Pierani et al., 2001).

The homeodomain transcription factors Evx1 is required to maintain the identity of the V0 population. In mice where Evx1 is knocked out, V0 neurons migrate in a ventrolateral direction, residing in the lateral ventral horn, and project axons ipsilaterally within the ventrolateral funiculus (Pierani et al., 2001). This new phenotype is characteristic of the V1 interneurons arising from the more ventral progenitor domain p1. The transfate phenotype observed in Evx1-knockout V0 neurons to a V1 identity highlights the importance of the homeodomain transcription factors that act to define a post-mitotic interneuron population.

The V0 population is heterogeneous. At least three subpopulations have been described – the excitatory (V0e), inhibitory (V0i), and cholinergic (V0c)

subpopulations. The V0 population as a whole plays important roles in establishing left-right alternation during walking (Lanuza et al., 2004). The Pitx2-expressing V0c do not migrate and reside around the central canal, with axons that project both ipsilaterally, as well as bilaterally, also synapsing directly onto motor neurons. These V0c neurons are involved in task-dependent swimming movements (Zagoraiou et al., 2009).

1.1.2. V1

The V1 interneurons arise from the Pax6/Dbx2/Nkx6.2-expressing p1 progenitor domain and transiently express the transcription factor Engrailed-1 (En-1) (Burrill et al., 1997; Matise and Joyner, 1997; Ericson et al., 1997; Sauressig et al., 1999; Vallstedt et al., 2001). Once these neurons become post-mitotic, between E8.5 to E12.5 (Alvarez et al., 2005), they begin a ventrolateral migratory route to settle within lamina IX and the lateral lamina VII (Alvarez et al., 2005; Sauerssig et al., 1999). The V1 population extends axons rostrally (Sauerssig et al., 1999) to form inhibitory synapses on motor neurons and other interneurons. The role of En-1 during development of the V1 population is to control axon fasciculation and proper pathfinding towards their motor neuron targets (Sauerssig et al., 1999). V1 neurons lacking En-1 form irregular fasciculations and a lack of motor neuron synapsing.

One important role of V1 INs in the lumbar motor system is to control the stepping speed of the animals (Gosgnach et al., 2006). Several subpopulations arise from the p1 progenitor domain, including Renshaw Cells (RCs) and Ia inhibitory interneurons

(IaINs). It has also been shown that the specialization of RCs and other V1 subpopulations is controlled by temporally restricted molecular events. RCs and IaINs are two of the best-characterized interneuronal types from their physiological properties and functions in the spinal cord. RCs receive strong input from motor neuron collaterals, and in turn inhibit those same motor neurons in order to fine-tune output. IaINs receive excitatory inputs from Ia sensory afferents and specifically inhibit motor neurons of antagonistic muscles. IaINs are also inhibited by RCs . This is a good example that functionally distinct interneurons can arise from a single progenitor domain.

1.1.3. V2

The Lhx3-expressing p2 progenitor domain, located just dorsal to pMN in the developing neural tube, generates V2 neurons between E10 to E13.5 (Ericson et al., 1997; Nardelli et al., 1999; Zhou et al., 2000; Smith et al., 2002; Peng et al., 2007). V2 neurons migrate in a lateral direction, distributing in a broad band through Lamina VII, from the central canal to the lateral edges of the grey matter (Lundfald et al., 2007). p2 gives rise to two broad categories of post-mitotic V2 interneurons (Peng et al., 2007): the Chx10 expressing excitatory V2a population and the Gata2- and Gata3-expressing inhibitory V2b population (Ericson et al., 1997; Zhou et al., 2000; Karunaratne et al., 2002; Smith et al., 2002). Recently, a third Sox1-expressing V2c subpopulation has been described, with more subpopulations suggested by genetic analyses (Panayi et al., 2010). V2a neurons are spread evenly throughout the band across Lamina VII, whereas V2b cells form more distinct

populations – one around the central canal (Lamina X), and another spanning Lamina VII (Lundfald et al., 2007). V2 neurons are ipsilaterally and caudally projecting interneurons. V2a show an interesting embryonic shift in their axon distribution. At E13.5, Chx10+ V2a project both rostrally and caudally or bifurcate for roughly 2 spinal segments. At birth (postnatal day (P) 0), however, the V2a population projects only caudally, suggesting a loss of their embryonic rostrally projecting axons (Lundfald et al., 2007).

V2a interneurons are known to play a role in both left-right coordination and the robustness of locomotor output (Crone et al., 2008). V2b may be involved in extensor and flexor coordination of the hind limb movements (unpublished, M Goulding).

1.1.4. V3

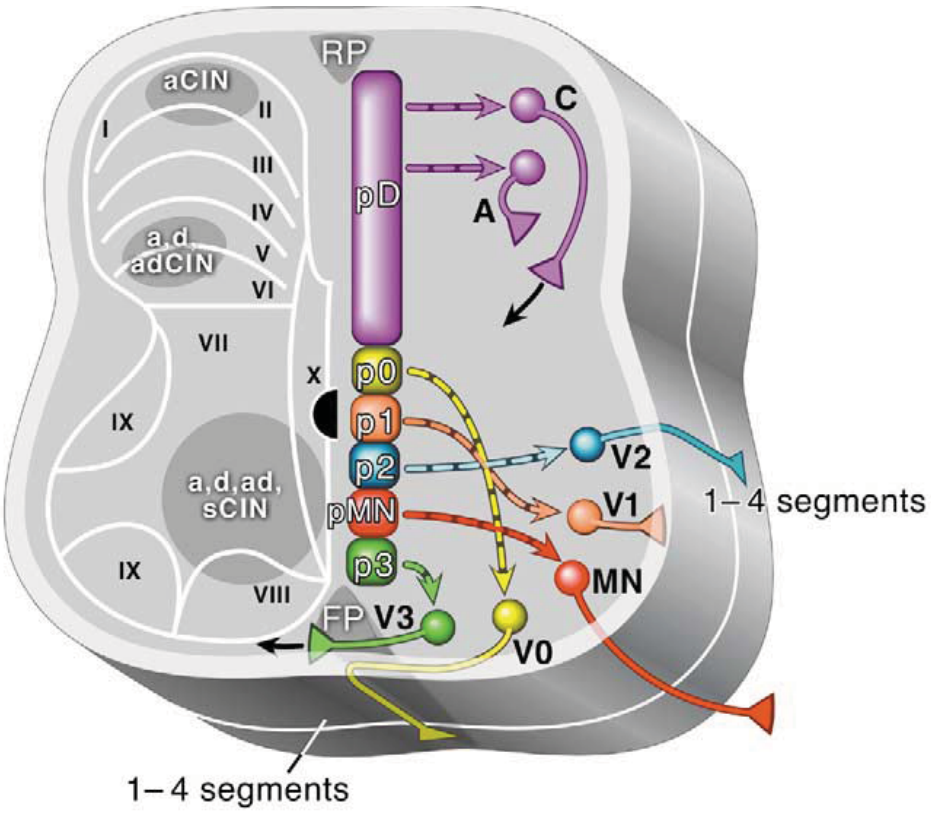
Compared to other ventral cell types, much less is known about the developmental events of V3 interneurons (INs). V3 INs emerge from the Nkx2.2/2.9-expressing p3 progenitor domain (Briscoe et al., 1999; Goulding et al., 2002), which is the ventral-most progenitor domain in the developing neural tube. V3 INs exit the progenitor domain between E9.0 to E12.0 and become post-mitotic (Jessel, 2000; Zhang et al., 2008). V3s express the transcription factor Single-minded 1 (Sim1) while entering the postmitotic stage, and continue expression until E16.5.

V3 are glutamatergic, predominately commissural interneurons that have been previously shown to synapse directly onto contralateral motor neurons, RCs, IaINs

and other interneurons (Zhang et al., 2008). It has been shown that V3 is required for the coherent, robust rhythmic bursting of motor neuron outputs during fictive locomotion. It also ensures a proper walking gait by balancing the activity of left and right halves of the spinal cord (Zhang et al., 2008).

V3 INs are present throughout the spinal cord, but the specific distribution in the lower thoracic and higher lumbar region of the mature mouse is distinct from the distribution at other spinal levels. Only in this region, V3 neurons occupy three areas along the ventro-dorsal axis, residing in lamina VIII, lamina VII, and lamina IV/V of the dorsal horn. The diverse distribution of V3 neurons suggests they play a variety of roles in the mature spinal cord.

Although the mature organization of V3, as well as its specific functioning within the hindlimb motor output, has been studied (Zhang et al., 2008), the developmental processes that occur during embryogenesis have yet to be established. It is these processes that provide an understanding of how the V3 INs are able to integrate themselves into the different lamina of the spinal cord, and become contributing members of the lumbar locomotor circuit. An understanding of the migration pathway and the axon growth of these unique interneurons will provide critical information not only about the spinal locomotor output, but also about neural development as a whole. One goal of my work was to investigate the developmental process of the V3 neurons.



Goulding and Pfaff, 2005

Figure 2. Schematic of spinal cord development.

The left half depicts the general location of commissural interneurons (CINs) within the different Rexed Lamina. Ascending (a), descending (d), ascending-descending (ad), and segmental (s) projection types are labeled. The right half depicts the origin of interneurons (IN) and motor neurons (MN), as well as their migratory route and type of axon projections. Adapted from Goulding and Pfaff, 2005.

1.2. Migration and Axon Guidance

Once neurons leave their progenitor domain and become post-mitotic, their task is to properly integrate themselves into appropriate neural networks. Two closely connected actions, migration and axon projection, lead to the neuron settling at the correct position and reaching the correct targets. The proper location of a neuron allows it to receive the proper synaptic inputs. The development of an axon that projects, ultimately synapsing on the correct target, allow the interneuron to relay the input received.

1.2.1. Migration

Using recombinant retrovirus, Leber and Sanes (1995) described the migratory patterns of neurons and glia in the embryonic chick spinal cord. They found that most spinal neurons exit the ventricular zone along radial glial cells in a process referred to as radial migration. These neurons then undergo a combined radial and tangential or just tangential migration along the dorso-ventral axis to their final position. The majority of neurons migrate ventrally from their original position, especially all cells from dorsal neural tube. Neurons migrating in a ventral to dorsal direction are a minority in the developing neural tube, and they all originate from the ventral progenitor domains, including motor neurons.

Motor neurons are born over a 24-hour period and migrate via radial migration (Wentworth, 1984; Leber et al., 1990; Leber and Sanes, 1995; Eide and Glover, 1996). The process of establishing medial (MMC) and lateral motor columns (LMC)

resembles the inside-out development of the cerebral cortex – with the LMC neurons migrating through the earlier-born MMC neurons (Hollyday and Hamburger, 1977). A second aspect of migration is tangential migration, which is responsible for the non-linear migration patterns. This allows for the more intricate distribution of spinal interneurons observed in the lumbar spinal cord.

Most interneurons in the spinal cord travel in a dorsal to ventral direction including V0, V1, and V2 interneuron populations (Moran-Rivard et al., 2001; Saueressig et al., 1999; Lundfald et al., 2007). These spinal interneurons appear to migrate along their axon trajectory. The commissural V0 neurons migrate in a ventromedial trajectory (Moran-Rivard et al., 2001), while both V1 and V2 ipsilateral populations project axons into the ventrolateral funiculus, and migrate along the same path (Saueressig et al., 1999; Lundfald et al., 2007). However, the V3 interneurons that originate from the most ventral progenitor domain can be found in different laminae from ventral to deep dorsal horn (Zhang et al., 2008), which indicate some V3s may migrate in a dorsal direction. In my study, I will follow the migratory path of these V3 neurons.

1.2.2. Axon guidance

Axonal outgrowth, path finding and target selection are important and complex processes that ensure that the axon reaches the correct target as well as help it position the cell body properly. The axonal outgrowth initially relies on the cues and substrates provided by the extracellular environment, and then it is further guided by a series of diffusible molecules. The first identified diffusible guiding molecules

were netrins, Netrin-1 and 2, secreted by the floorplate and lower region of the spinal cord, respectively. The gradient of Netrin-2 and Netrin-1 promote the axons of dorsal commissural neurons to outgrow and travel ventrally to reach the floor plate and cross the midline. (Tessier-Lavigne et al., 1988; Kennedy et al., 2006; Serafini et al., 1994; Kennedy et al., 1994; Serafini et al., 1996). It has been shown that the attractive response of developing axons to the high Netrin-1 concentration is mediated by the Deleted in Colorectal Cancer (DCC) receptor (Keina-Masu et al., 1996), with possible assistance of the Down Syndrome Cell Adhesion Molecule (DsCAM) receptor to help push the axons into and through the midline (Ly et al., 2008; Liu et al., 2009). Interestingly, when Netrin-1 was knocked out in a mouse model, all commissural neurons in the spinal cord, with the exception of V3, lost their ability to cross the midline. This produces a coordinated, although only synchronous, gait in the adult mouse (Rabe, et al., 2009). In contrast to this, mice with the DCC knockout (Netrin-1 receptor) lost crossing of V3 axons – the only difference between the Netrin-1 and DCC knockout phenotypes. In contrast to the synchronous gait of the Netrin-1 knockout, the DCC knockout mouse results in a complete loss of coordination between the left and right sides (Bernhardt et al., 2012). This difference indicates that DCC might be a receptor for another, yet to be discovered, diffusible molecule involved in pre-crossing axon guidance.

In addition to Netrin-1, it has recently been shown that Shh, which also is present at high concentration at the floor plate, also plays an important role in the

development of dorsal commissural axons. (Charron et al., 2003; Sanchez-Camacho and Bovolenta, 2009).

Although Netrin-1 and Shh promote a developing axon to move ventrally, commissural axon guidance towards the ventral midline also receives a repulsive signal from the dorsal roof plate. BMP7 and growth/differentiation factors 7 (GDF7) were found to be strong repellents to the initiation of the ventral trajectory of commissural axons (Augsburger et al., 1999; Butler and Dodd, 2003; Yamauchi et al., 2008). The protein Draxin (dorsal repulsive axon guidance protein) is also thought to play a role, although a much smaller one, and may be involved in axon fasciculation (Islam et al., 2009).

The distinction between ipsilateral and commissural axons is dependent on the Robo family of receptors, which are present on the developing axon growth cone (Kidd et al., 1998; Battye et al., 1999; Kidd et al., 1999). These receptors respond to the repulsive effect of Slit proteins, which are present at the midline of the developing neural tube.

Robo1 is utilized in both Drosophila and vertebrates to establish an ipsilateral axon projection phenotype (Kidd et al., 1998; Zou et al., 2000; Long et al., 2004; Chen et al., 2008). Downregulation of this receptor via *commissureless* (*comm*) in the Drosophila and Robo3.1 in vertebrates decreases the sensitivity of a developing axon to repulsive Slit signals at the midline, and produces a commissural phenotype

(Bonkowsky et al., 1999; Georgiou et al., 2002; Keleman et al., 2002; Hiramoto and Hiromi., 2006; Chen et al., 2008; Sabatier et al., 2004).

It was suggested that once an axon crosses over the midline, there is both a loss of attraction (Shirasaki et al., 1998) and a gain of repulsion (Zou et al., 2000) to the Shh and Netrin-1 gradients that initially were attractive (Lyuksyutova et al., 2003). After midline crossing, a commissural axon becomes sensitive to Slit proteins, which prevent axons from crossing the midline a second time (Zou et al., 2000). These axons also become sensitive to the repulsive cues of the Semaphorin (Sema) family, particularly Sema3B and Sema3F (Zou et al., 2000). This change in repulsion is thought to "switch on" through interaction with high levels of Shh present at the floor plate (Para and Zou, 2010; Zou et al., 2000). This change in sensitivity is the result of an altered transcription factor profile and different ligand receptors being expressed on the developing axon growth cone.

The turning of an axon either rostrally or caudally is a decision that must be made by both ipsilateral and contralateral projections alike. Two gradients, Wnt and Shh, are both present in longitudinal gradients that are responsible for this directional change in vertebrate axons (Lyuksyutova et al., 2003). Shh is present in a longitudinal gradient that is high caudally and low rostrally, and acts largely as a post-crossing repulsive agent for commissural axons (Bourikas et al., 2005). This Shh gradient indirectly "carves out" an opposite caudal-low, rostral-high attractive

Wnt gradient through the expression of the Wnt-inhibiting Sfrp-1/2 (Lyuksyutova et al., 2003; Domanitskaya et al., 2010).

Until now, most studies have focused on the dorsal commissural neurons, and little is known about the axonal trajectories of ventrally originated commissural neurons, particularly V3s. One goal of my study was to understand the developmental stages of V3 axon projections, and the projection profile of this population of interneurons in the newborn spinal cord.

1.3. Sim1

The transcription factor Sim1 is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors initially found in Drosophila (reviewed in Kewley, 2004). The Sim1 transcription factor is conserved across several species, and has homologues in mouse, zebrafish, Drosophila, and humans. The mouse version of Sim1 acts as a repressor protein when heterodimerized with ARNT (Ema et al., 1996; Moffett, Reece, and Pelletier, 1997; Michaud et al., 2000, Moffett and Pelletier, 2000; Woods and Whitelaw, 2002). Sim1 is expressed in many regions of developing mouse CNS, including mamillary bodies (MB), hypothalamus, and spinal cord (Xu et al., 2003; Marion et al., 2005; Zhang et al., 2008).

Sim1 begins to be expressed in the neuroepithelium that gives rise to the Mammillary bodies (MB) of the hypothalamus at around E9.5 (Marion et al., 2005). In the development of this region, Sim1 is known to be critical in proper formation of the mammilothalamic tract (MTT), connecting the MB with the thalamus, and the

mammilotegmental tract (MTEG), connecting the MB to the tegmentum (Marion et al., 2005), both of which are ipsilateral tracts. In mice where Sim1 has been knocked down, these two tracts are not only hypo-developed, but are seen to aberrantly cross the midline (Marion et al., 2005). The MBs in Sim1 knockout animals show upregulation of Rig-1/Robo3 expression. This ligand/receptor combination functions in a cell autonomous fashion to inhibit the midline-repulsion of Slit signaling in neurons on the CNS (Sabatier et al., 2004; Marillat et al., 2004). It is therefore suggested that Sim1 plays a role in downregulating Rig-1/Robo3, which maintains the ipsilateral projections seen in the MTT and MTEG.

The role of Sim1 in axon guidance is also seen in the development of the hypothalamus-spinal cord (HTS) projections in zebrafish (Schweitzer et al., 2013). When Sim1a (or its binding partner Arnt2) is knocked out, there is a medial shift of the HTS projections. The proposed downstream effecter is also implicated to be Robo3a.1. Loss of Sim1a causes increased expression of Robo3a.1, which leads to down-regulation of Robo2, and loss of sensitivity to the inhibitory Slit signals at the midline. The end result is midline attraction of the HTS.

Sim1 has also been implicated in neural migration defects, observed in the development of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the mouse hypothalamus (Xu et al., 2003). Although Sim1 knockout has no effect on generation of cells within the PVN or SON, they were unable to migrate to their proper location, and located to an intermediate position between the normal PVN

and SON (Xu et al., 2003). It was suggested that the downstream factor to Sim1 might be the receptor-like molecule Plexin-C1 (Xu et al., 2003).

It was also shown that Sim1 mutant cells might be less likely to undergo programmed cell death than cells with a functional Sim1 gene (Xu et al., 2003).

As mentioned earlier, p3 progenitor neurons in the spinal cord start expressing Sim1 while entering the postmitotic state and Sim1 becomes the molecular marker that defines the V3 interneuron population. However, the function of Sim1 as a transcription factor in the developing spinal cord is still largely unknown. The current study was the first attempt to investigate the role of Sim1 during the development of V3 interneurons in the spinal cord.

Spinal cord development is an intricate process that yields a remarkable system producing coordinated locomotion. The project described in this thesis addressed three main points:

1. Anatomically characterize the V3 population of interneurons through embryonic stages and in the newborn spinal cord.
2. Establish the axon projection profile of V3 neurons within the lumbar spinal cord of newborn mice.
3. Understand the role of Sim1 in the development of V3 interneurons.

Chapter 2. Materials and Methods

2.1. Animals

Mouse strains

Generation of *Sim1*$^{Cre/+}$ and *Sim1*$^{tLacZ/+}$ mice were described in Zhang et al (2008). *Rosa 26tdTom* mice were kindly provided by Dr. Hongkui Zeng (of the Allen Institute, also available at the Jackson Laboratory stock 007908). *Sim1*$^{Cre/+}$*::tdTom* mice were generated by crossing the two strains. The *Sim1* knockout mouse was produced by crossing *Sim1*$^{Cre/+}$*::tdTom* with *Sim1*$^{tLacZ/+}$ to generate *Sim1*$^{Cre/tLacZ}$*::tdTom*. All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the University Committee on Laboratory Animals at Dalhousie University.

The presence of a vaginal plug the next morning confirmed fertilization, and this time point was defined as embryonic day 0.5 (E0.5).

Prior to surgery, pregnant mice were injected with a mixture of ketamine (60 mg/kg) and xylazine (12 mg/kg). Mouse embryos with placenta were then delivered by cesarean section. After removal of all the mouse embryos, the anesthetized mouse was euthanized by cervical transection.

Immediately after delivery, the embryos were placed in Ringer's Solution that contained the following (mM): 6.49 NaCl; 0.23 KCl; 1.98 D-Glucose; 2.1 $NaHCO_3$; 0.31 $MgSO_4$; 0.37 $CaCl_2$; 0.15 KH_2PO_4. The solution was constantly bubbled with 95%

O2/5% CO2 to maintain a pH of 7.4. *Sim1:Cre::tdTom* positive embryos were identified by the expression of red fluorescent proteins. Tracing procedures were carried out on the lumbar segment of the spinal cord of mouse embryos aged E12.5, E14.5, and P0.

2.2. Spinal Cord Dissection

During dissection, the embryos were kept in oxygenated (95% O2/5% CO2) Ringer's Solution renewed every 5–10 minutes to maintain oxygenation and pH buffering. Each embryo was pinned out in a supine position in a dissection tray. The thorax and abdomen were cut open on both sides of the midline, and the embryo was eviscerated. The vertebral column was cleared of any tissue and the vertebral bodies were removed by laminectomy. In P0 mice, the spinal roots were cut and the spinal cord was removed. In embryos, the spinal cord was left in the body during the incubation to prevent possible destruction to the spinal cord.

2.3. Application of Biotin-linked Dextran Amine

3 kDa biotin-linked dextran amine was dissolved in a small drop (1 μL) of distilled water on a plastic petri dish under a dissection microscope. Small drops of the dextran solution were collected on the tip of a needle (BD PrecisionGlide, 0.45mm x 10mm) and formed crystal beads. At the desired level, a small cut was made through one half of the spinal cord. Care was taken not to cross the midline with the incision. The dextran crystals were inserted at the cut sites and taken up by the cut axons. After dextran application the preparation was not moved for 10-15 minutes

to allow as much tracer as possible to be taken up. The preparations were incubated at room temperature, in the dark, in constantly oxygenated Ringer's Solution. Incubation time ranged from 6 to 20 hours, increasing with embryonic stage (younger embryos required less incubation time). After incubation, the spinal cords were pinned on a dissection plate and fixed using 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline (PBS), pH 7.4 for 1-3 hours.

2.4. Sectioning

Tissue was removed from fixative and immersed in 20-30% sucrose in PBS overnight. Excess tissue was removed from around the spinal cord and the tissue was placed in a plastic mould containing O.C.T. compound (Tissue-Tek, Torrence, Ca) and frozen using dry ice and ethanol. Preparations were stored at -20 degrees or for longer-term at -80 degrees Celsius.

The spinal cords were sectioned transversely at 20 – 30 μm, depending on age of the spinal cord, on a cryostat at -17 degrees Celsius. Serial sections were collected on Fisher Super Frost slides (Superfrost Plus microscope slides – Fisherbrand, USA). Slides were dried at room temperature in a dark box for 2 hours before being stored in the dark at -20 degrees Celsius.

2.5. Immunohistochemistry and Histochemistry

Immunohistochemistry was performed on spinal cord cross sections using rabbit anti-tdTom primary antibody (Clontech). The secondary antibody was a goat anti-rabbit IgG (Invitrogen).

Sections were first washed in PBS for 5 minutes before being washed in PBS containing 0.1% Triton X (PBST) 3 times, each for 5 minutes. Slides were incubated in blocking solution (PBS containing 10% goat serum (Invitrogen) and 0.1% Triton X) for 1 hour at room temperature to block non-specific binding. Slides were then incubated overnight at room temperature with the primary antibody (in blocking solution) at a concentration of 1:1000. The primary antibody used was rabbit anti-tdTom. Slides were washed 3 times, each for 5 minutes in 0.1% PBST before being incubated in Alexa Fluor 569-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, inc.) secondary antibody diluted in 0.1% PBST. Secondary antibody concentration was 1:500. Alexa Fluor 488-conjugated streptavidin (Jackson ImmunoResearch Laboratories, inc.) was added, when required to label biotin-linked dextran amine, at this stage and incubated for the 2 hours. Slides were then rinsed 3 times for 5 minutes with PBS before being cover slipped over mounting media (Dako fluorescent mounting medium). Stained slides were stored at 4 degrees Celsius.

Images were captured using a Zeiss Axiovert 200M inverted fluorescence microscope. The *tdTom* and dextran double-labeled neurons were counted and

digitally mapped on representative cross-sections using an assigned grid to ensure accurate positioning. This grid separated one half of the spinal cord cross section into 3 strips vertically and 4 strips horizontally, forming 12 boxes. Specific analysis of each experiment will be further described in the Results sections.

2.6. Statistics

Student's *t*-test (two-tailed) was used to determine statistical significances between *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ V3 cell counts. Significance is indicated as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Data in results is mean ± SD.

V3 projection analysis followed Poisson distribution (Rosner, 2011) with the standard deviation (S) calculated as S = sqrt (mean/n). The approximate 95% confidence intervals was estimated as 1.96*S, which was used to compare the difference of projecting V3s between *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ spinal cords. Comparisons with non-overlapping approximate 95% confidence intervals were deemed significantly different ($p \leq 0.05$).

Chapter 3. Results

3.1. V3 neurons show a unique dorsolateral migration pattern

It has previously been found that V3 neurons have a broad distribution in the postnatal spinal cord, and may represent spatially and functionally separate subpopulations (Zhang et al., 2008; Borowska et al., unpublished). However, it is not clear how these subpopulations develop during embryonic stages. My first study was to investigate the developmental pattern of V3 neurons at different stages of embryogenesis.

The main tool in this study was a transgenic mouse line, *Sim1Cre:tdTom*. This mouse line includes two modified genes: the first encodes Cre recombinase encorporated into the first coding exon of *Sim1* using standard homologous recombination. The second is a *LoxP*-flanked STOP sequence in front of the *tdTom* gene inserted in the *Rosa-26* locus. This Cre-loxP combination results in the specific expression of tdTom fluorescent proteins only in the *Sim1*-expressing V3 neurons. While Cre is expressed under the *Sim1* promoter, this recombination event does not demonstrate perfect efficacy. The possibility does exist for either ectopic expression of tdTom protein in non-V3 interneurons, or the absence of Cre recombination in *Sim1* expressing V3 interneurons. While I acknowledge the possibility that V3 neurons could be missed, or non-V3 neurons could be counted, the tdTom positive cells predominately are V3 neurons (Zhang et al., 2008). To avoid any confusion and complication, throughout the thesis I will still refer to $tdTom^+$ cells as V3 neurons.

We first observed cells with tdTom fluorescence in the spinal cord of *Sim1Cre:tdTom* mice at E10-10.5 (Fig 3A.), which is slightly later than the presumed beginning of *Sim1* expression, likely due to the time gap between the transcription of *Cre* and the expression of the tdTom protein. At E10.5-11.5, V3 neurons all cluster immediately above the floor plate, when observed on a cross section of the spinal cord (Fig 3A, B.), but at E12.5 some of these cells begin a possible combination of radial and tangential migration in the dorsal and lateral direction at all spinal segments analyzed (Fig 3C-C''.). In E13.5 spinal cords, the dorsal tangential migration continues and some V3 neurons have reached the dorsal half of the spinal cord. However, a group of V3 neurons lags behind in the ventral position, which shows the initial signs of V3 neuron separation into subgroups (Fig 3D-D''.). At the same time, V3 neurons also start showing different migration patterns along the rostro-caudal spinal cord. The V3 neurons in the Lower Thoracic and Higher Lumbar segments travel further in the dorsal direction than cells in other segments (Data not shown for cervical and sacral regions). Such separation becomes obvious at E14.5. At this stage, Ventral V3s are clearly separated from the dorsally migrating Non-Ventral subpopulation (Fig 3E-E''.). At the Higher Lumbar region an additional subpopulation can be observed as well. The distinct fates of these E14.5 subpopulations are made clearer in the E15.5 spinal cord. While the Lower Thoracic and Lower Lumbar regions retain a Ventral and Non-Ventral subpopulation (Fig. 3F, F''), the Higher Lumbar region reveals 3 distinct tdTom positive populations, which can be given designations as Ventral, Dorsal, or Intermediate (Fig 3F'.), based on

their locations. The P0 spinal cord reveals the final locations of V3 neurons along the ventro-dorsal axis. Both Lower Thoracic (Fig 3G. T12-13) and Higher Lumbar (Fig 3G'. L1-4) regions contain three subpopulations: Ventral (Lamina VIII), Intermediate (Lamina VII), and Dorsal (Lamina IV, V). The distribution of V3 neurons within the Lower Lumbar region (Fig. 3G''. L4-6) is less easily determined, but can be separated as either Ventral or Non-Ventral subpopulations. At all three spinal levels analyzed, the Ventral population constitutes the highest number of V3 neurons present (Lower Thoracic = apprx. 60%, Higher Lumbar = apprx. 52%, Lower Lumbar = apprx. 71%).

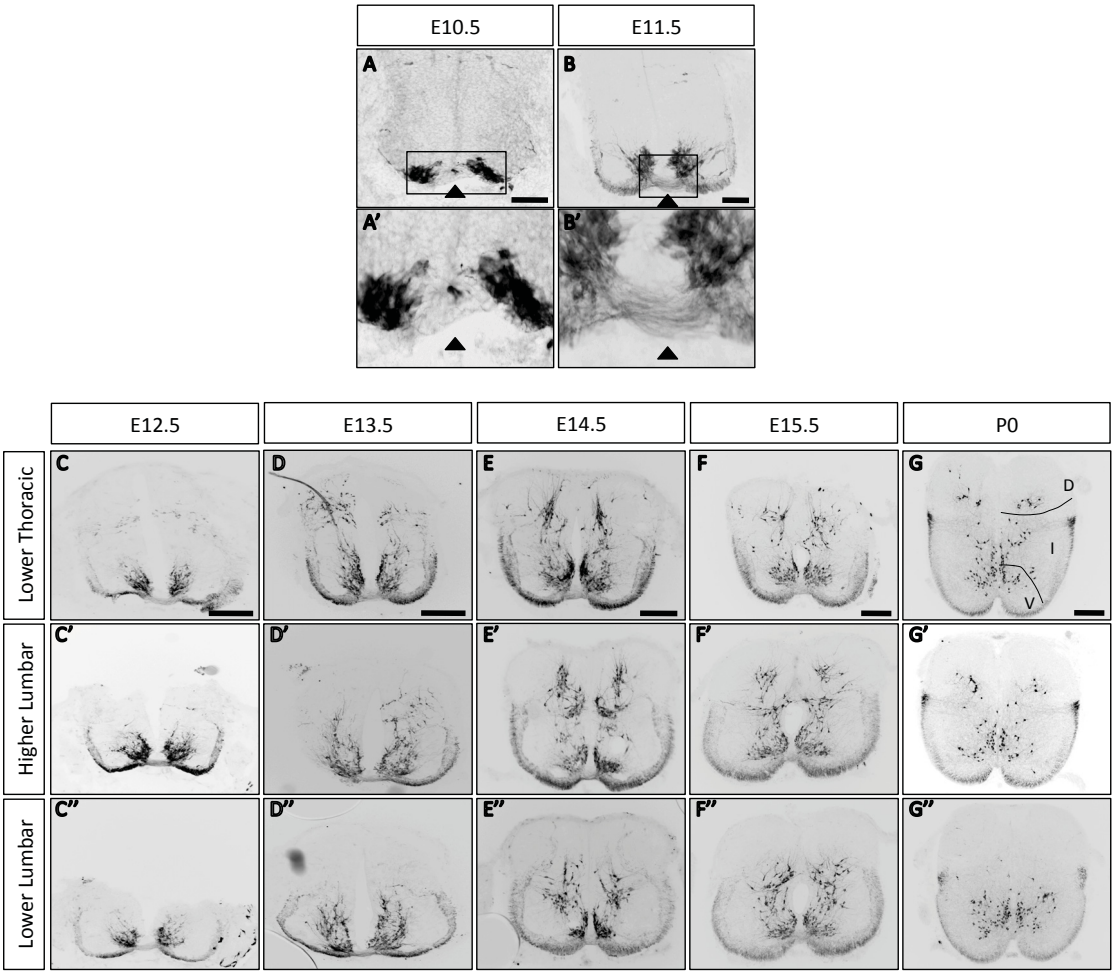

E10.5
E11.5
A
B
A'
B'
E12.5
E13.5
E14.5
E15.5
P0
Lower Thoracic
Higher Lumbar
Lower Lumbar
C
D
E
F
G
C'
D'
E'
F'
G'
C''
D''
E''
F''
G''
D
I
V

Figure 3. *Sim1*$^{+}$ V3 neurons arise from the ventral-most progenitor domain and migrate in a dorso-lateral direction.

A – B'. Emergence of post-mitotic V3 interneurons and midline crossing, visualized by the expression of tdTom. V3 neurons emerge from their progenitor domain at E10.5 (A), however they have not extended axons across the ventral commissure (A'). At E11.5, V3 neurons continue to emerge from the progenitor domain (B), and have begun sending axons across the midline (B'). Arrows indicate the ventral commissure. (Scale bar = 100μm)
C – G''. Representative images of spinal cord cross sections from different embryonic stages. V3 neurons begin their dorsolateral migration at E12.5 (C – C''), with the initial indication of two migratory streams. E13.5 spinal cord cross sections showing continued dorsolateral migration of V3 neurons, and initial indication of ventral and non-ventral subpopulations (D – D''). E14.5 spinal cord cross section (E – E'') showing two clear subpopulations in the Low Thoracic (E) and Low Lumbar (E'') spinal cord. The High Lumbar section (E') shows a ventral subpopulation and two separate non-ventral populations. Cross sections from an E15.5 spinal cord (F – F'') also show two subpopulations in the Low Thoracic (F) and Low Lumbar (F'') region. The High Lumbar region (F') shows 3 subpopulations, separated into relative ventral, intermediate, and dorsal locations. P0 spinal cord cross sections (G – G'') show 3 subpopulations in the Low Thoracic (G) and High Lumbar (G') region. Only 2 subpopulations are seen at the Low Lumbar level (G''). V = Ventral, I = Intermediate, D = Dorsal. (Scale bar = 200μm)

3.2. V3 neurons show a transient ipsilateral projection during development

A previous study revealed that V3 neurons are predominately commissural in adulthood (Zhang et al., 2008). In my current study, V3 axons were also detected crossing the midline in the ventral commissure at E11.5 (Fig. 3B, B'). To further reveal the axon projection patterns of V3 neurons during development, we conducted retrograde tracer studies using 3kDa biotin-dextran amine. Following axon cutting, the dextran amine is taken up and transported from axon to soma using the intrinsic axonal transport machinery. We applied dextran-amine crystals on one side of the isolated spinal cord at either the L1 or L5 region (Fig. 4a).

The spinal cords of different embryonic stages are drastically different in length, and therefore require different incubation times in order to allow for adequate travel of the dextran-amine dye. E12.5 spinal cords were incubated in continuously oxygenated Ringer's solution for 6-8 hours, while E14.5 and P0 spinal cords were incubated overnight, all at room temperature. After incubation, the labeled spinal cords were transversely cryosectioned and dextran-amine was visualized using Alexa 488-conjugated Streptavidin (Fig 4b). Dextran-amine tract tracing is an extremely variable procedure that is dependent on maintaining healthy neural tissue for dye transport. We therefore set standards for acceptable dye transport in order to constitute an adequate representation of axonal projections. E12.5 spinal cords required 1000 μm of dextran transport to be considered acceptable, E14.5 spinal cords required 1500 μm, and P0 spinal cords required 2100 μm; all of which cover at least 3 segments at the different ages (Nissen, 2005).

Spinal cords were serially cryosectioned using 10 slides. This meant that individual sections on a single slide were separated by either 200 μm or 300 μm, depending on the section thickness. For projection analysis, 4 non-consecutive slides were selected and analyzed for each spinal cord (40% of the total cord). Neurons that were double-labeled with both dextran-amine and tdTom were counted and mapped on transverse sections of the spinal cord. Each double labeled neuron was also digitally mapped on a representative spinal cord cross section for analysis along the ventro-dorsal axis. We denoted the site of dextran crystal insertion as 0. Negative distances represent neurons projecting caudally to the cut site and are termed descending V3s (dV3s), and positive distances represent neurons projecting rostrally to the cut site and are termed ascending V3s (aV3s) (Fig. 5, 6, 7, 12, 13, 14, 15).

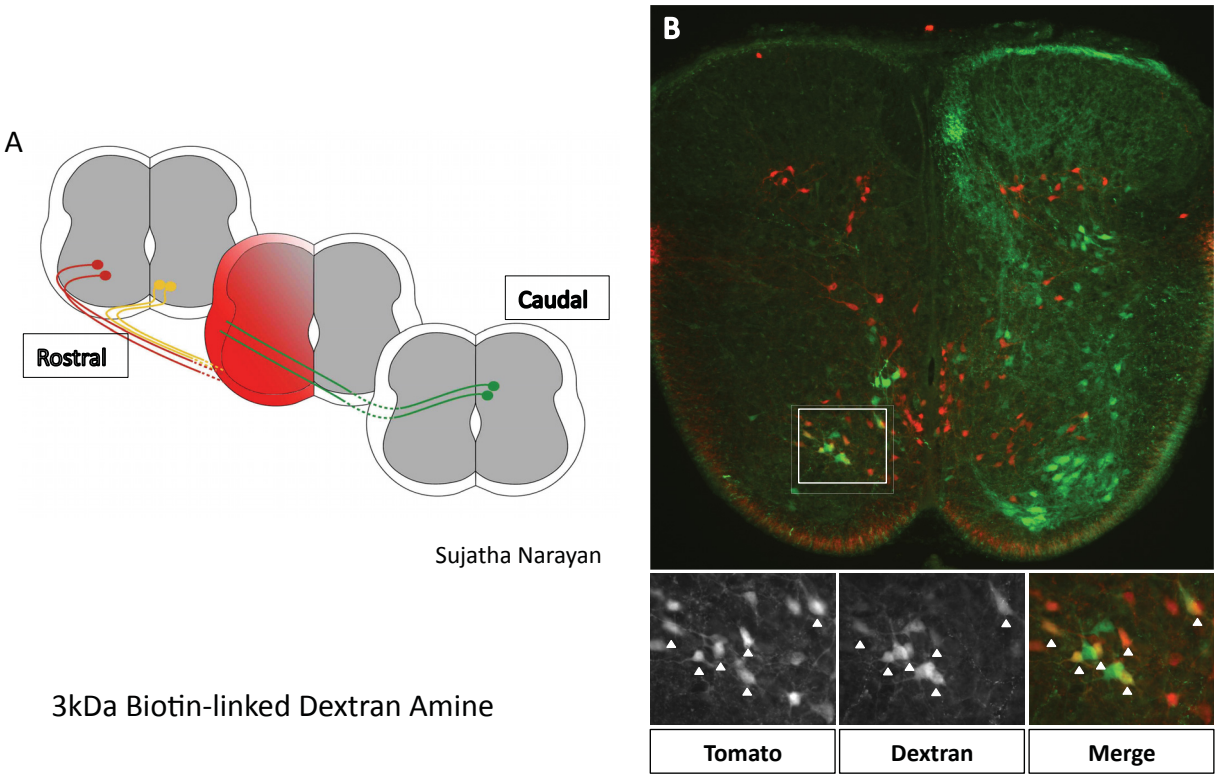


Figure 4. Biotin-linked dextran amine labels axon projections in a retrograde fashion.

A. Schematic diagram of the biotin-linked dextran amine tract tracing protocol, indicating retrograde labeling.
B. Representative image of a spinal cord cross section after dextran amine tract tracing and conjugated with Streptavitdin-488. Arrows indicate V3 neurons double labeled with tdTom and dextran amine.

The V3 projection pattern in spinal cords changes during postmitotic development. At E11.5, V3 neurons have started to send axons across the midline. A day later at E12.5 we could detect a group of dextran-labeled V3 neurons on the contralateral side to the dye injection site – roughly L1. At the same time, however, we also observed a similar number of ipsilaterally projecting V3s (contralateral = 32±4 cells per cord, ipsilateral = 38±6 cells per cord; n=2). More interestingly, the ipsilateral V3s contained predominately descending projections (~97%, Fig. 5A), while the contralaterally projecting V3s were mainly ascending (~ 84%, Fig. 5B). This result indicates at this stage there are at least two main subpopulations of V3 – one with descending ipsilateral projections (diV3s) and another with ascending contralateral projections (acV3s). The diV3s and acV3s show similarities and differences in distribution in the dorsoventral axis (Fig. 5C, D). Both types show one cluster in the ventromedial area of the spinal cord cross section, close to the p3 progenitor domain, and a second cluster more dorsolateral. While the ventromedial populations of ipsilateral and contralateral projections are intermingled at this stage, the dorsolateral populations contain ipsilateral projections and are located in a more lateral position than contralateral projections (Fig. 5C, D).

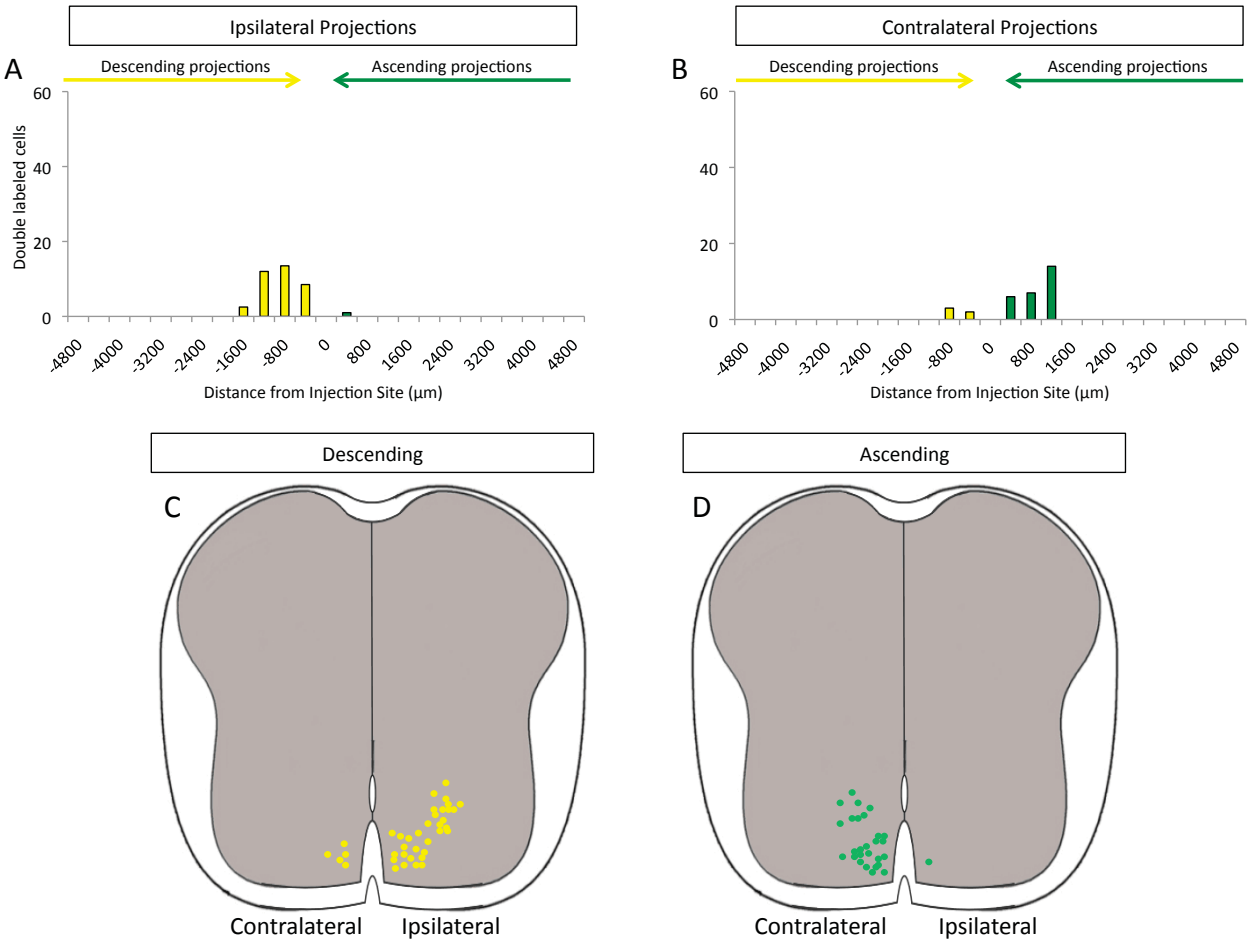

Ipsilateral Projections
A
Descending projections
Ascending projections
Double labeled cells
60
40
20
0
-4800
-4000
-3200
-2400
-1600
-800
0
800
1600
2400
3200
4000
4800
Distance from Injection Site (µm)
Contralateral Projections
B
Descending projections
Ascending projections
60
40
20
0
-4800
-4000
-3200
-2400
-1600
-800
0
800
1600
2400
3200
4000
4800
Distance from Injection Site (µm)
Descending
C
Contralateral
Ipsilateral
Ascending
D
Contralateral
Ipsilateral

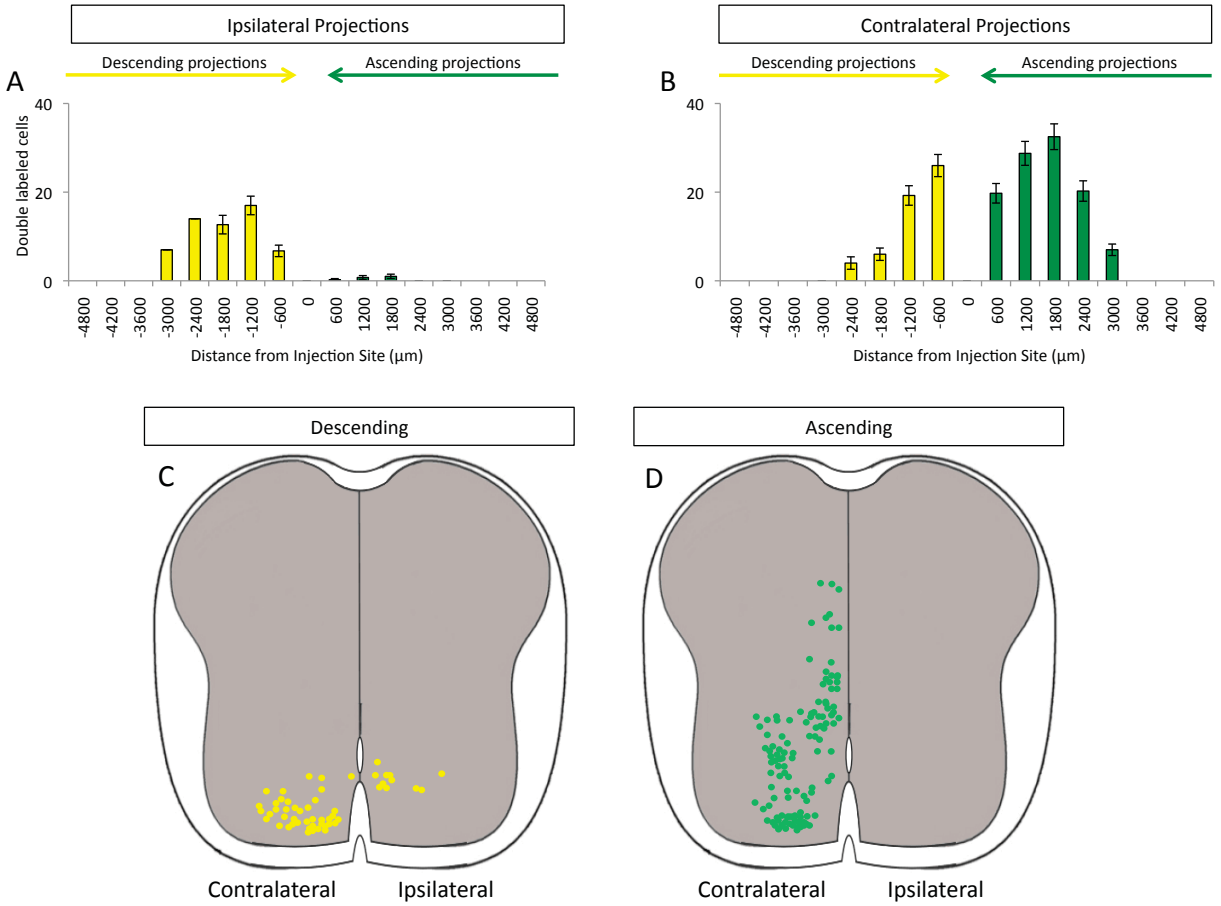
Figure 5. At E12.5 V3 neurons contain ipsilateral and contralateral projections.

A, B. Quantification of Sim1^{+} V3 neurons in the E12.5 embryo retrogradely labeled with biotin-linked dextran amine. 0 on the X-axis (approximately L1) marks the site of tracer injection, with negative numbers representing distance from the site of tracer injection (μm) of V3 soma with descending projections, while positive numbers represent V3 soma with ascending projections. The majority of ipsilateral projections (A) are descending (yellow bars). Ascending projections (green bars) are mostly commissural (B) (n=2).
C, D. Projection map illustrating the ventro-dorsal soma position of V3 neurons in the E12.5 embryo with descending (C) and ascending (D) projections to the site of dextran injection. The descending projections (yellow dots) show a similar distribution to the ascending projections (green dots) in the ventromedial region of the spinal cord cross section. Diagrams are composites of 20 μm sections of a single representative spinal cord.

At the E14.5 stage, the projection pattern shows a change in the distribution of V3 axons (Fig. 6). The number of dextran positive V3s increased at all segments both rostral and caudal to the site of injection, on both ipsilateral and contralateral sides of the spinal cord. The contralaterally projecting cells, however, did become more dominant. When we compared the analyzed cords from E12.5 and E14.5, there is a 3-fold increase in the total number of double labeled V3s. However, the diV3s increased by only around 36% in the E14.5 spinal cord, while the acV3s increased 4-fold between this time period. A third projection population, the descending commissural V3s (dcV3s) appeared at E14.5, representing one third of commissural V3s. Interestingly, most of this 'new' population was detected close to the injection site, suggesting they might be in the earlier stages of developing their axons.

Overall, at E14.5 around 51% of all double labeled cells were acV3, while approximately 26% were dcV3, and approximately 23% were ipsilateral projecting V3s, most of which were descending (diV3s). (Fig. 6A, B; n=5).

The V3 neurons with different projections had distinctive distribution patterns on the cross section of the spinal cord (Fig. 6C, D). DcV3s were almost exclusively located in the ventromedial area of the E14.5 spinal cord (Fig. 6C), whereas acV3s were more widely distributed along the dorsoventral axis (Fig. 6D). A ventromedial population of acV3s is present, along with medially and laterally positioned groups migrating dorsally. The diV3s are clustered in a medial location, partially intermingled with the dcV3 population (Fig. 6C).


Ipsilateral Projections
Descending projections
Ascending projections
A
Double labeled cells
40
20
0
-4800
-4200
-3600
-3000
-2400
-1800
-1200
-600
0
600
1200
1800
2400
3000
3600
4200
4800
Distance from Injection Site (µm)
Contralateral Projections
Descending projections
Ascending projections
B
40
20
0
-4800
-4200
-3600
-3000
-2400
-1800
-1200
-600
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600
1200
1800
2400
3000
3600
4200
4800
Distance from Injection Site (µm)
Descending
C
Contralateral
Ipsilateral
Ascending
D
Contralateral
Ipsilateral

Figure 6. V3 projections at E14.5 contain descending ipsilateral projections, as well as ascending and descending contralateral projections.

A, B. Quantification of *Sim1*$^{+}$ V3 neurons in the E14.5 embryo retrogradely labeled with biotin-linked dextran amine. 0 on the X-axis (approximately L1) marks the site of tracer injection, with negative numbers representing distance from the site of tracer injection (μm) of V3 soma with descending projections, while positive numbers represent V3 soma with ascending projections. Ipsilateral projections (A) are almost exclusively descending (yellow bars). Contralateral projections (B) are both descending and ascending (green bars) (n=5).
C, D. Projection map illustrating the ventro-dorsal soma position of V3 neurons in the E14.5 embryo with descending (C) and ascending (D) projections to the site of dextran injection. Descending contralateral projections are more ventral than the descending ipsilateral projections, which are located near the central canal (yellow dots). Ascending projections are located contralateral to the site of dextran injection, and are distributed along the ventrodorsal axis (green dots) of the spinal cord cross section. Diagrams are composites of 30 μm sections of a single representative spinal cord.

The changes in the projection pattern of V3s continued to P0. As we describe in the previous section, at P0, spinal V3s distribute as clusters at ventral, intermediate and dorsal regions in the cross sections of Lower Thoracic and Higher Lumbar segments, while only Ventral and Non-Ventral subgroups are clearly distinguishable in other segments. Furthermore, length of the spinal cord has increased substantially from E14.5 to P0.

The most noticeable change at birth is that ipsilaterally projecting V3s become sparse, with V3s become predominately commissural neurons (~97% cV3s) (Fig. 7A, B). The distribution of the commissural neurons, however, is similar to E14.5 with both acV3 and dcV3 present at P0, although the peak of the descending population has moved away from the immediate cut site, which is different from what is shown at E14.5 (Fig. 7B).

To obtain a detailed projection profile of these V3 neurons, in addition to the group with the L1 application site, we also applied dextran at the L5 region of a separate group of P0 spinal cords (Fig. 8).

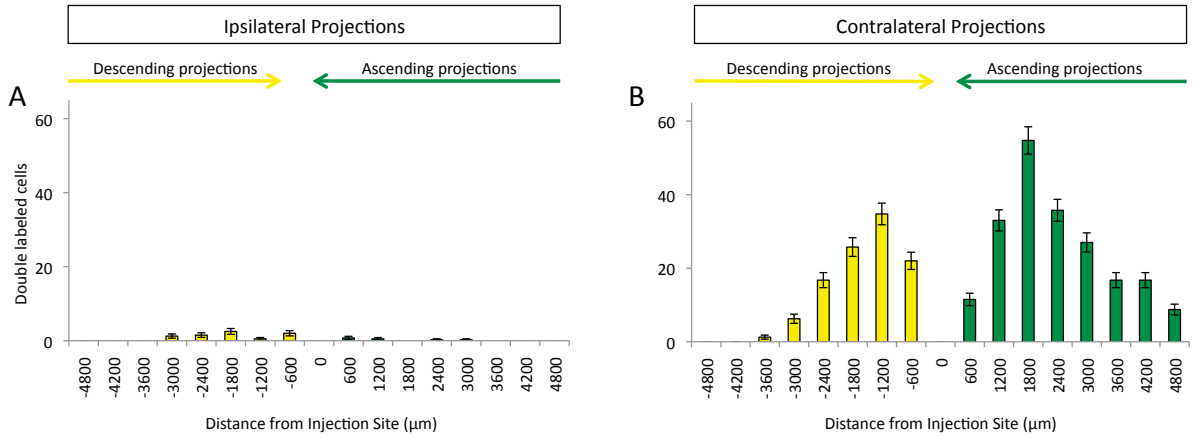


Figure 7. V3 neurons in the P0 spinal cord are almost exclusively commissural.

A, B. Quantification of *Sim1*$^{+}$ V3 neurons in the P0 spinal cord retrogradely labeled with biotin-linked dextran amine. 0 on the X-axis marks the site of tracer injection, with negative numbers representing distance from the site of tracer injection (μm) of V3 soma with descending projections, while positive numbers represent V3 soma with ascending projections. Ipsilateral projections (A) are almost completely absent, with the majority of V3 neurons having contralateral projections (B) in both the descending (yellow bars) and ascending (green bars) direction (n=4).

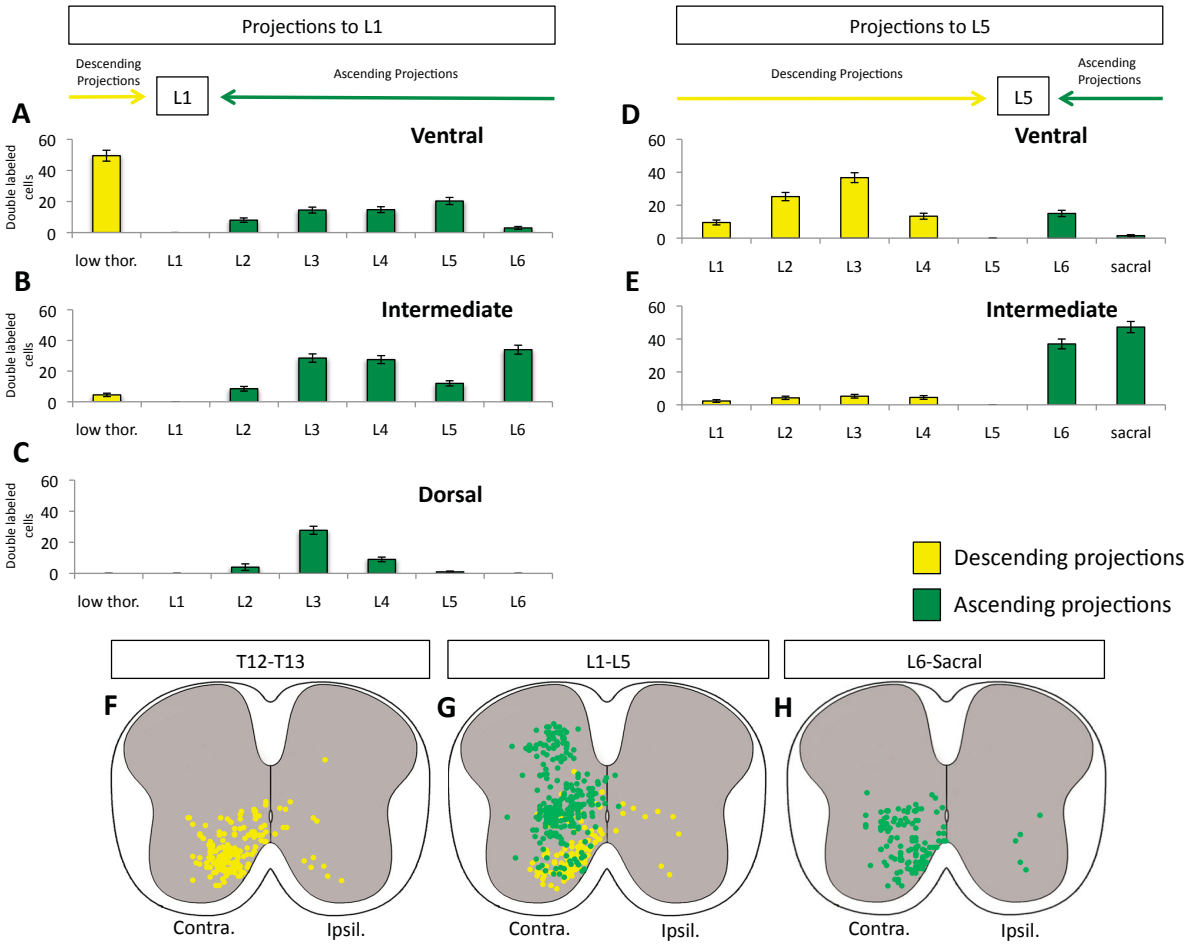
More detailed analyses were conducted with both L1 and L5 application sites (Fig 8), in order to understand the extent of V3 axon connection between these two spinal cord segments, which are considered to represent flexor (L1) and extensor (L5) innervations. We constructed the diagrams of the dextran positive V3 cells of each subpopulation at Lower Thoracic and each lumbar segment (Fig. 8A-E) as well as their distribution maps on cross sections (Fig. 8F-H). For each spinal cord, four of 10 sections were analyzed, and a total of 20 sections for Lower Thoracic and 12 sections for each Lumbar segment were grouped together to identify the number of projecting cells in that segment.

In all lumbar segments, Ventral V3s were a mixed population that could send descending axons to L5 or ascending axons to L1 (Fig. 8A, D), and they could reach up to at least 4 segments in each direction. Using our current method, however, we were not able to identify if any of these neurons were bifurcating. Overall, in the lumbar region, there was more descending Ventral V3s than ascending ones (65±8 and 58±8 cells per cord, respectively). The majority of descending V3s reaching the L5 region were from L2 and L3 (Fig. 8D), while V3s ascending to L1 were more evenly distributed (Fig. 8A).

The Intermediate population, on the other hand, was composed of mainly ascending V3s with a small fraction of descending cells (13±4 for descending verses 86±10 for ascending V3s, Fig. 8B, E). It is interesting that there may be two peaks of Intermediate V3s with ascending projections to L1, at L3-4 and L6 (Fig. 8B).

Compared to Ventral V3s, the Dorsal V3s in the lumbar region were more homogenous with only ascending axons. The majority of Dorsal V3s reaching L1 were located in the L3 segment (Fig. 8C).

Since we applied dextran at L1 and L5, we could only detect descending V3s at Lower Thoracic segments and ascending V3s in the L6/Sacral region. Similar to the lumbar segments, descending V3s in the thoracic segments are predominately ventral cells (~92%, Fig. 8A). While it was a mix of Ventral and Intermediate populations with ascending axons in the sacral region, the majority is Intermediate V3s (~ 64%, Fig. 8D, E).


Projections to L1
Descending Projections
L1
Ascending Projections
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Ventral
Double labeled cells
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L1
L2
L3
L4
L5
L6
B
Intermediate
C
Dorsal
Projections to L5
Descending Projections
L5
Ascending Projections
D
Ventral
sacral
E
Intermediate
Descending projections
Ascending projections
T12-T13
L1-L5
L6-Sacral
F
G
H
Contra.
Ipsil.

Figure 8. V3 subpopulations show differential projection patterns at P0.

A, D. The Ventral subpopulation shows the most diverse projection pattern of all V3 subpopulations. They show descending projections (yellow bars) from the Lower Thoracic to L1, ascending (green bars) and descending projections within the Lumbar region to L1 and L5, and ascending projections from L6/Sacral to L5.
B, E. The Intermediate subpopulation contains mostly ascending projections to both L1 and L5, with a small number of descending projections visible.
C. The Dorsal subpopulation contains exclusively ascending projections to L1 (n=4).
F-H. Summary schematic showing the dorso-ventral position of descending (yellow dots) and ascending (green dots) V3 projections located within the Lower Thoracic (T12-T13, F), the Lumbar (L1-L5, G), and the L6/Sacral (H) region. Diagrams are composites of 30 μm sections of a single representative spinal cord.

3.3. *Sim1* is required for proper migration and axon outgrowth of V3 interneurons

The Sim1 transcription factor is specifically expressed in spinal V3 neurons during most of their postmitotic period (E9.5-16, unpublished observations), but its function in the development of V3s is still unknown. To determine its function, we used Sim1 knockout mice compared to their heterozygous littermates. We interbred *Sim1Cre:tdTom* , which has Cre recombinase knocked into the *Sim1* locus, and *Sim1:taulacZ*, which has a TaulacZ coding sequence inserted into the first coding exon of the *Sim1* gene. This cross was able to produce both Sim1 heterozygotes ($Sim1^{+/cre}$) and Sim1 knockouts ($Sim1^{tLacZ/cre}$) in the same litter. As described by previous findings (Marion et al., 2005) mice with *Sim1* knocked out cannot survive past a few hours post-natally but *Sim1* knockout mice at P0 did not show obvious physical deformities or behavior defects compared with control littermates. Identification of heterozygotes and knockout mice was done using post-mortem genetic analysis.

tdTom is still used to trace V3 neurons in both $Sim1^{+/cre}$ and $Sim1^{tLacZ/cre}$ spinal cords. As described for $Sim1^{+}$ neurons in other regions of the CNS (Xu et al., 2007; Marion et al, 2005), when *Sim1* is knocked out, neuron identity does not change. In our study, $Sim1^{tLacZ/cre}$ spinal cords have normal V3s emerging from the p3 progenitor domain at E10.5, and their axons started crossing the ventral commissure at E11.5

(data not shown). However, the migration and projection patterns of V3 neurons are significantly different between the *Sim1* knockouts and their littermate controls.

3.3.1. *Sim1*$^{tLacZ/cre}$ V3 neurons show a deficient migration path

As described previously, although V3 neurons started showing dorsal migration at E12.5, they only clear separated into different clusters of cells at E14.5, especially the appearance of the dorsal subpopulation in the Lower Thoracic and Higher Lumbar regions (fig. 9A-B'). At this stage, however, the *Sim1*$^{tLacZ/cre}$ spinal cord failed to show any clear clustering, even though V3 neurons still dispersed along the dorsoventral axis. This migration defect was seen more clearly in the Higher Lumbar region of the *Sim1*$^{tLacZ/cre}$ spinal cord, where V3 neurons failed to fully migrate to a dorsal position, and did this not form the three distinct clusters seen in *Sim1*$^{+/cre}$ spinal cords (Fig. 9A' to B'). Migration of the V3 neurons in the Lower Lumbar region is not as extensive as that observed in the Lower Thoracic and Higher Lumbar regions. No migratory defects were seen in either the *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ in this region (fig. 9A'', B'').

At P0, unlike the three distinct V3 subpopulations seen along the dorsoventral axis of the Lower Thoracic and Higher Lumbar regions in *Sim1*$^{+/cre}$ spinal cords, V3 neurons in the *Sim1*$^{tLacZ/cre}$ spinal cords had a few scattered cells in the dorsal region, but an enlarged intermediate population (Compare Fig. 9C to 9D, and 9C' to 9D').

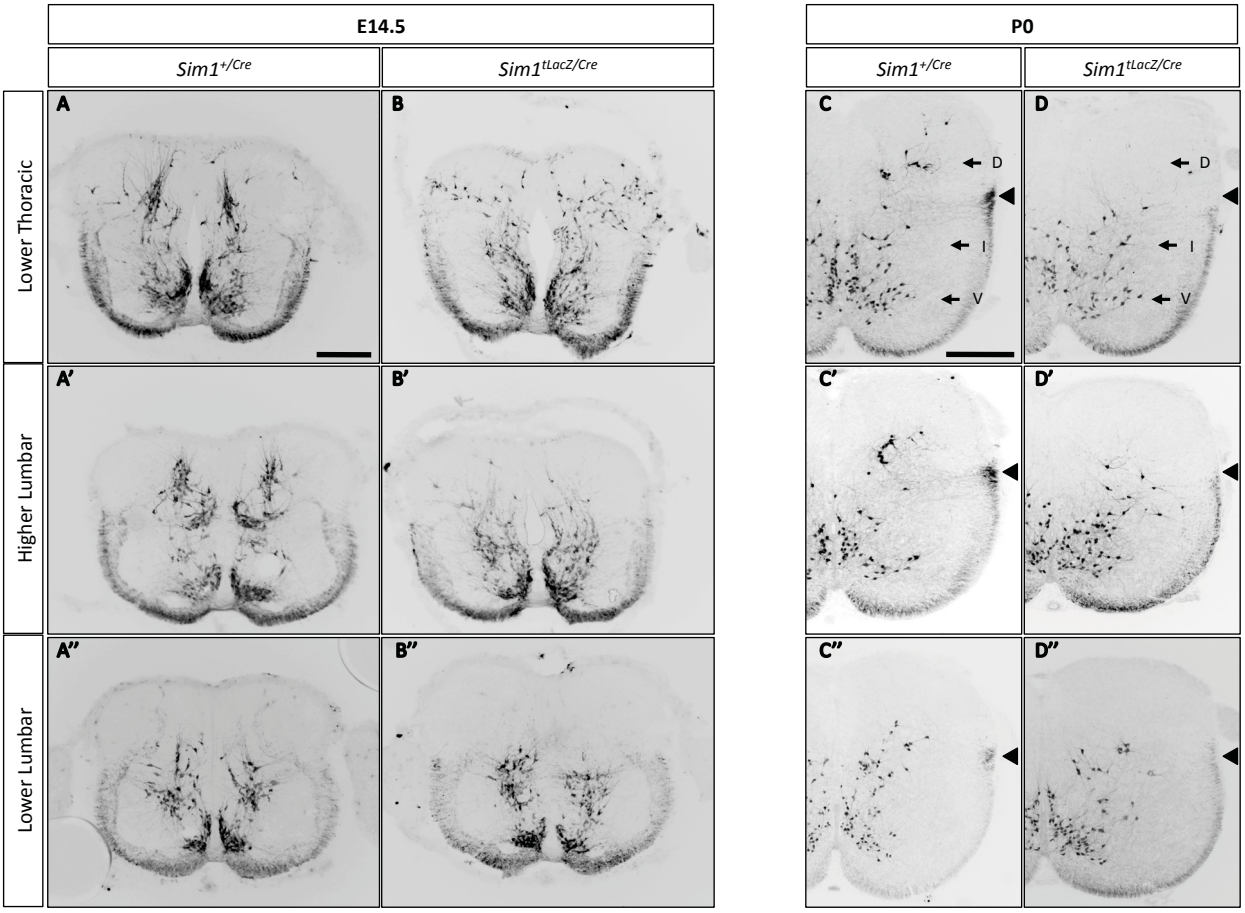
E14.5
P0
Sim1+/Cre
Sim1tLacZ/Cre
Sim1+/Cre
Sim1tLacZ/Cre
Lower Thoracic
Higher Lumbar
Lower Lumbar
A
B
C
D
A'
B'
C'
D'
A''
B''
C''
D''
D
I
V
D
I
V

Figure 9. *Sim1*$^{tLacZ/cre}$ V3 neurons show an altered migration trajectory.

A – B''. V3 neurons in the E14.5 spinal cord show absence of clustering and dorsal migration. V3 neurons in the low thoracic region of the *Sim1*$^{tLacZ/cre}$ spinal cord lack cohesive organization (compare A with B). This lack of cohesive organization is more apparent in the Higher Lumbar region of the E14.5 spinal cord (compare A' with B'). A migratory defect is not observed in the Lower Lumbar region (compare A'' with B''). (Scale bar = 200μm)
C – D''. V3 neurons show an altered distribution in the P0 spinal cord. The *Sim1*$^{tLacZ/cre}$ spinal cord shows a diminished Dorsal subpopulation with an increased Intermediate subpopulation in both the Lower Thoracic (compare C with D) and Higher Lumbar (compare C' with D') segments. The Lower Lumbar region does not show a migratory defect (compare C'' with D''). Arrows indicate Ventral (V), Intermediate (I) and Dorsal (D) subpopulations. Arrowheads indicate a lateral *Sim1*$^{+}$ tract that is absent in the *Sim1*$^{tLacZ/cre}$ P0 spinal cord. (Scale bar = 200μm)

We counted V3 neurons in *Sim1*$^{tLacZ/cre}$ and control spinal cords. We focused on the Lower Thoracic and Higher Lumbar regions, where there are distinctive Ventral, Intermediate, and Dorsal subpopulations (Fig 4G). For each spinal cord, two of 10 slides were used. For the Lower Thoracic, a total of 10 sections per spinal cord (5 sections per slide) were analyzed (covering 1500 μm), while 6 sections per spinal cord (3 sections per slide) were analyzed for each Lumbar segment (covering 900 μm). Each section was analyzed individually to determine the number of V3 neurons present within each subpopulation. The result is summarized in Fig 10.

The Ventral subpopulation had no difference in the number of cells between the *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ spinal cords at all spinal levels analyzed (Fig. 10A). Differences in V3 neuron distribution were seen in the Intermediate and Dorsal subpopulations of *Sim1*$^{tLacZ/cre}$ spinal cords at each spinal level analyzed. There was an increased number of V3 neurons located within the Intermediate region (Fig. 10B), and a decrease in the number of V3 neurons located in the Dorsal region (Fig. 10C). For example, in the L1 segment there was a 35% increase in Intermediate V3s in each 30μm section (*Sim1*$^{+/cre}$ = 15±4, n=24, *Sim1*$^{tLacZ/cre}$ = 23±4, n=30, $p \leq 0.001$) and a 45% decrease in Dorsal V3s in each 30 μm section (*Sim1*$^{+/cre}$ = 9±4, *Sim1*$^{tLacZ/cre}$ = 5±3, $p \leq 0.001$).

Analysis of the total number of V3 neurons within each spinal level revealed another interesting finding (Figure 10D). Spinal cords lacking *Sim1* had more V3 neurons than the control spinal cords (*Sim1*$^{+/cre}$: L.T., n=40, L1-L6, n=24; *Sim1*$^{tLacZ/cre}$: L.T.,

n=50, L1-L6, n=30). The increase in V3 numbers was seen most drastically in the Low Thoracic (~10% increase, $p \leq 0.05$), L3 (~15% increase, $p \leq 0.01$) and L4 (~10% increase, $p \leq 0.05$) spinal segments.

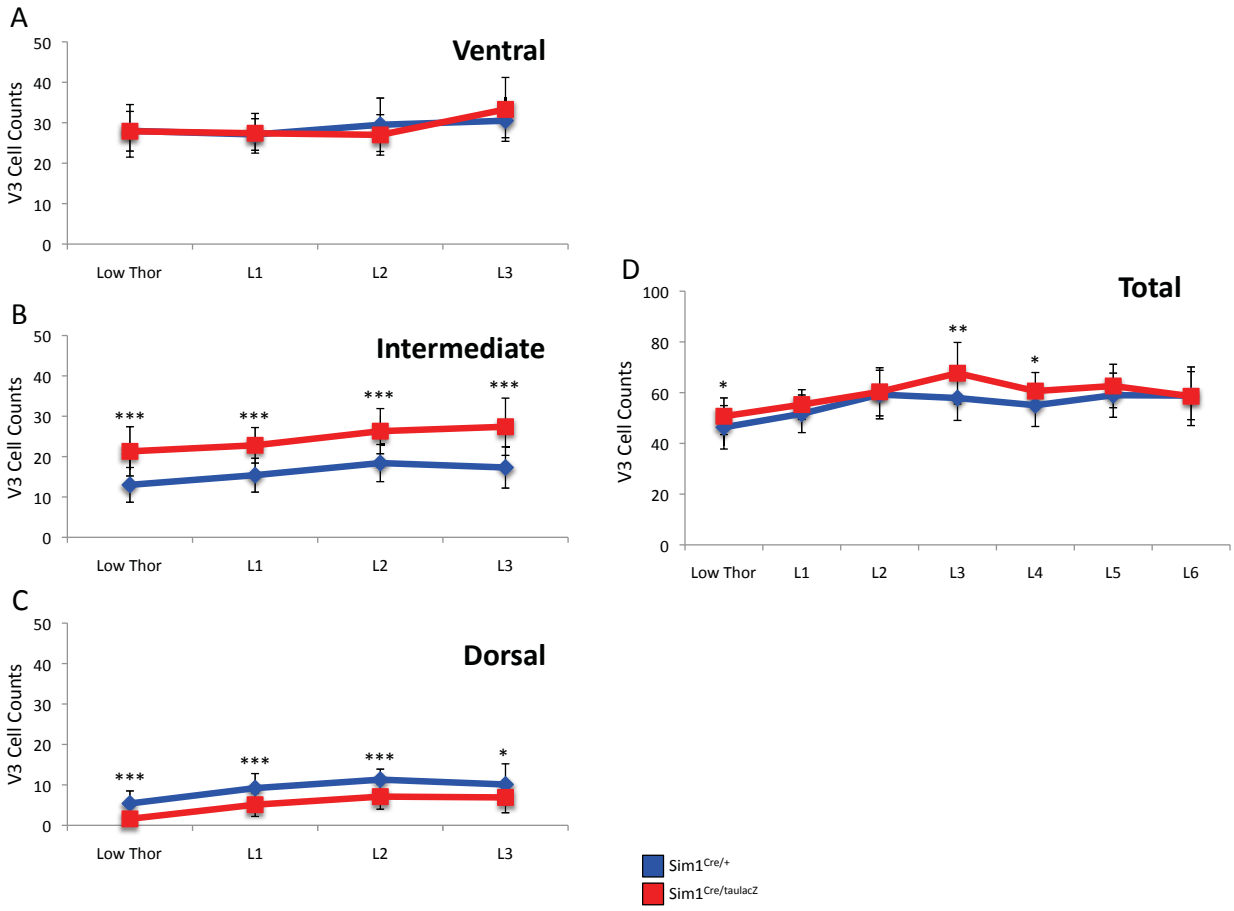

Figure 10. Dorsal *Sim1*$^{tLacZ/cre}$ V3 neurons incorrectly locate to their position, residing in the Intermediate region.

A – C. Cell counts of V3 neurons in 30 µm sections. No change was seen in the number of Ventral V3 neurons (A). *Sim1*$^{tLacZ/cre}$ spinal cords contained increased numbers of Intermediate V3 neurons (B) (L.T. – L3, $p \leq 0.001$). *Sim1*$^{tLacZ/cre}$ spinal cords show decreased numbers of Dorsal V3 neurons (C) (L.T. – L2, $p \leq 0.001$; L3, $p \leq 0.05$).
D. Total cell counts of V3 neurons, in 30 µm sections of the P0 spinal cord, at each spinal segment (Low Thoracic – L6). (*Sim1*$^{+/cre}$: L.T., n=40; L1-L6, n=24. *Sim1*$^{tLacZ/cre}$: L.T., n=40; L1-L6, n=30) (L.T., $p \leq 0.05$; L3, $p \leq 0.01$; L4, $p \leq 0.05$).

3.3.2. *Sim1* does not exclusively define a commissural phenotype in V3 neurons

Previous findings have shown that *Sim1* also plays a role in axon growth in the hypothalamus and other regions in the CNS (Marion et al., 2005; Schweitzer et al., 2013). Therefore, we next analyzed the projection pattern of V3 neurons in the $Sim1^{tLacZ/cre}$ spinal cord, using dextran retrograde tracing.

At E14.5, there was no difference in ipsilaterally projecting V3s between the spinal cords of $Sim1^{+/cre}$ and $Sim1^{tLacZ/cre}$ mice (Fig. 11A). Although there was also no significant difference in descending contralateral projections, there were significantly fewer ascending contralateral projections ($Sim1^{+/cre}$ = 115±9, n=5; $Sim1^{tLacZ/cre}$ = 69±9, n=3; $p \leq 0.05$) (Fig. 11B). The ventro-dorsal distribution of projecting V3 neurons revealed a lack of dorsal population in the $Sim1^{tLacZ/cre}$ spinal cords, which was consistent with the migration defects described above (Fig. 11C-F).

At this stage in development, the distribution of V3 neurons throughout the Lower Thoracic and Lumbar regions are easily separated into either Ventral or Non-Ventral designations, based on their position in the spinal cord cross section. Separating the V3 population into these two subpopulations provided further insight into where a potential axon projection deficiency is most prominent. When we divided the dextran positive commissural V3s into Ventral and Non-Ventral

subpopulations (Fig. 12), the Ventral V3 neurons in *Sim1*$^{tLacZ/cre}$ spinal cords displayed fewer projections in both ascending (*Sim1*$^{+/cre}$ = 42±6; *Sim1*$^{tLacZ/cre}$ = 27±6; $p \leq 0.05$) and descending (*Sim1*$^{+/cre}$ = 55±6; *Sim1*$^{tLacZ/cre}$ = 32±6; $p \leq 0.05$) directions (Fig. 12A). The similar decrease in projections was shown by the ascending non-ventral V3s (*Sim1*$^{+/cre}$ = 76±8; *Sim1*$^{tLacZ/cre}$ = 52±8; $p \leq 0.05$). Surprisingly, there was an increase in descending Non-Ventral V3 projections (*Sim1*$^{+/cre}$ = 5±2; *Sim1*$^{tLacZ/cre}$ = 13±4; $p \leq 0.05$), which may be due to the irregular distribution of V3 neurons in the *Sim1* knockout mouse (Fig. 12C, D). The projection deficiencies noticed at E14.5 indicate that V3 neurons that lack *Sim1* may not be able to properly project their axons, with deficiencies and differences noted in both Ventral and Non-ventral subpopulations.

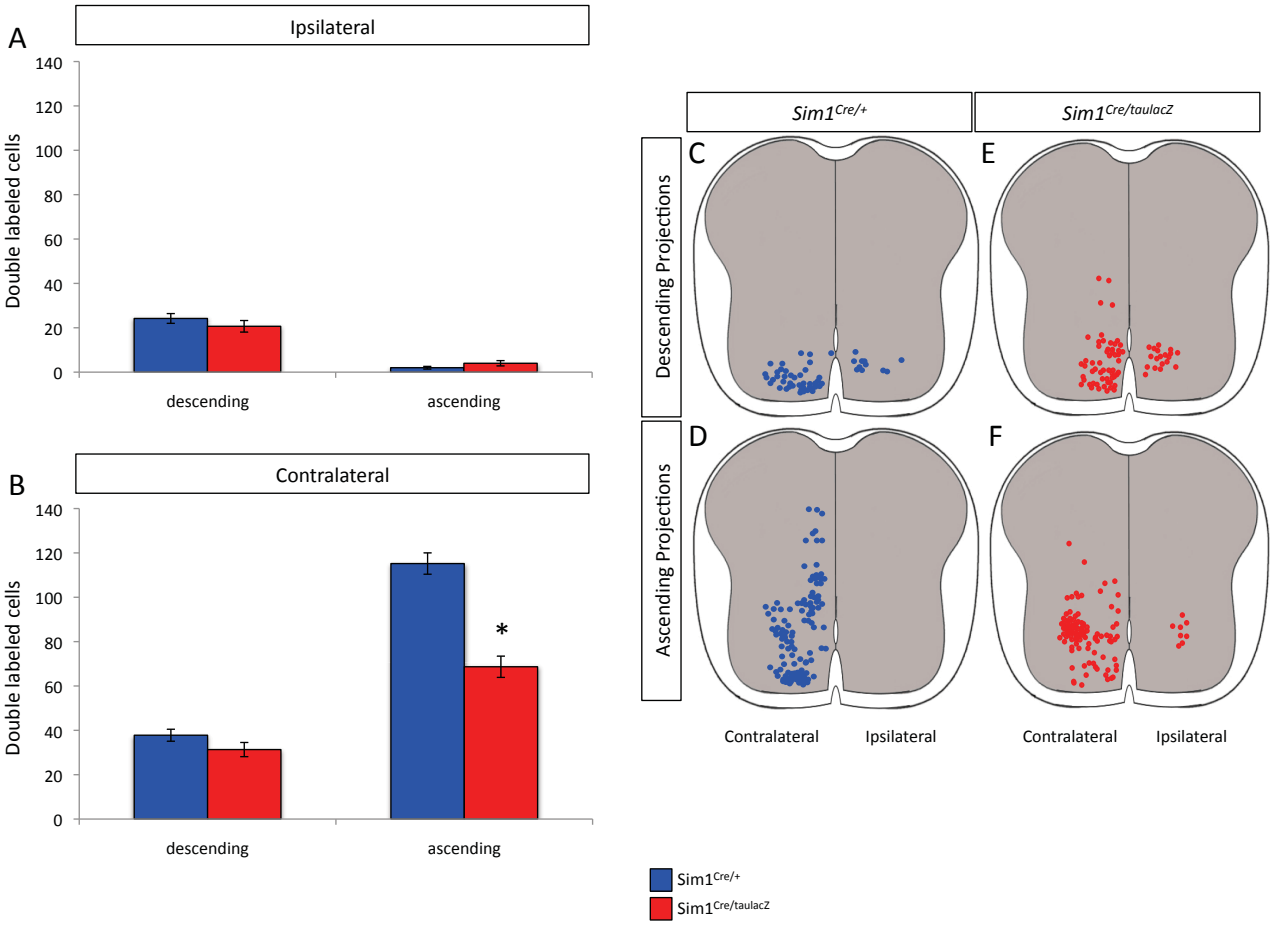
A
Ipsilateral
Double labeled cells
140
120
100
80
60
40
20
0
descending
ascending
B
Contralateral
Double labeled cells
140
120
100
80
60
40
20
0
*
descending
ascending
Sim1Cre/+
Sim1Cre/taulacZ
Descending Projections
Ascending Projections
C
D
E
F
Contralateral
Ipsilateral
Contralateral
Ipsilateral
Sim1Cre/+
Sim1Cre/taulacZ

Figure 11. Loss of *Sim1* does not affect ipsilateral or contralateral projections at E14.5.

A, B. Quantification of total Sim1$^+$ V3 neurons in *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ E14.5 embryos retrogradely labeled with biotin-linked dextran amine at L1. V3 neurons are labeled as either ascending or descending. Projections are either ipsilateral (A) or contralateral (B) to the site of dextran-amine injection (*Sim1*$^{+/cre}$, n=5; *Sim1*$^{tLacZ/cre}$, n=3; $p \leq 0.05$). The entire extent of dextran transport was analyzed.
C – F. Projection map illustrating the ventro-dorsal soma position of V3 neurons in the *Sim1*$^{+/cre}$ (blue dots) and *Sim1*$^{tLacZ/cre}$ (red dots) E14.5 embryo. Descending projections in the *Sim1*$^{tLacZ/cre}$ cross section show the presence of a more widespread Ventral subpopulation (E). Ascending projections in the *Sim1*$^{tLacZ/cre}$ cross section show a limited location, clustering in a more intermediate position compared with the larger distribution of the *Sim1*$^{+/cre}$ cross section. Diagrams are composites of 30 μm sections of a single representative spinal cord.

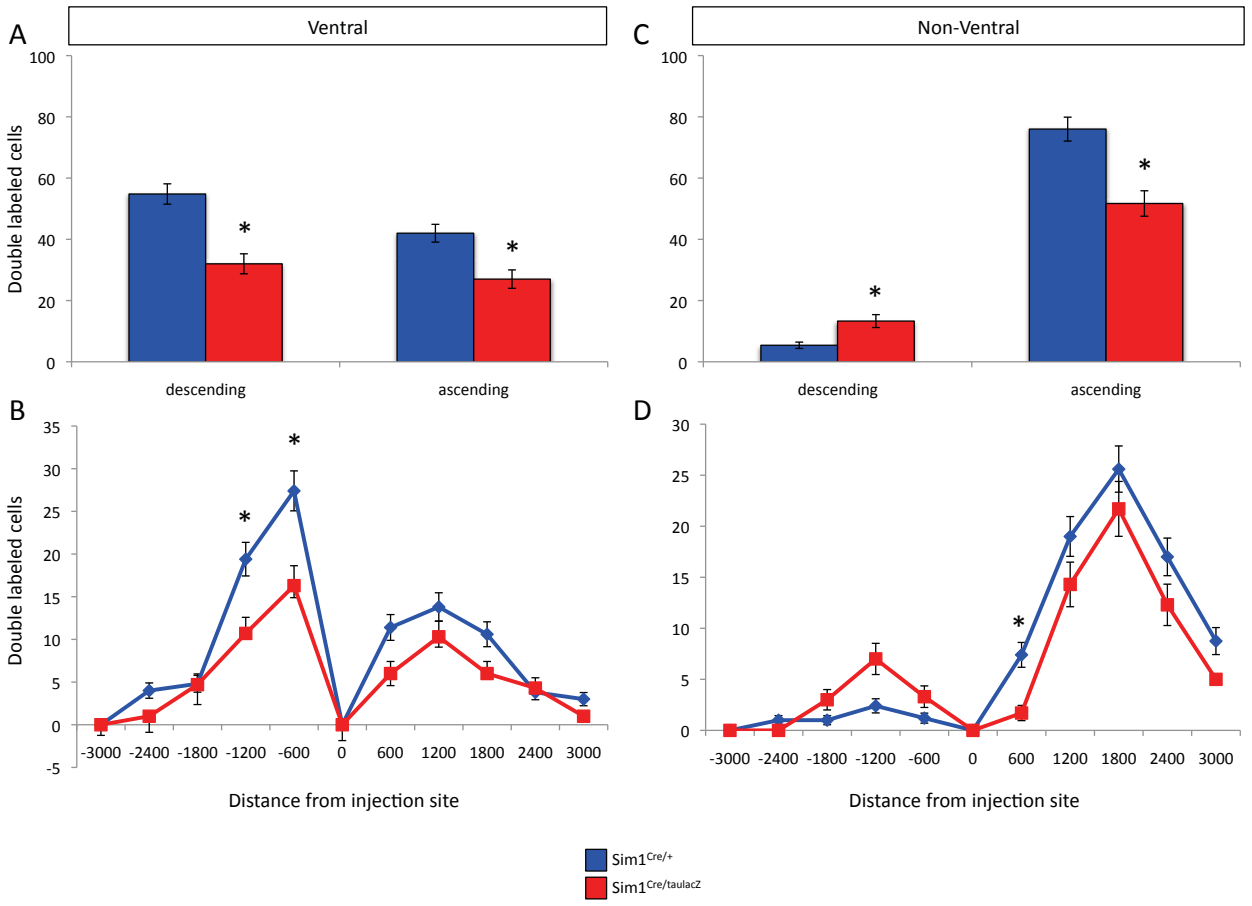

Figure 12. Loss of *Sim1* shows differences in axon projections of both Ventral and Non-Ventral subpopulations at E14.5.

A – D. Quantification of total ascending and descending axon projections of Ventral and Non-Ventral V3 interneurons in E14.5 *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ spinal cords when biotin-linked dextran amine is injected at L1. Ventral V3 neurons show a significant decrease in both ascending and descending projections, observed in the total counts (A), as well as the distance plots (B). Non-Ventral V3 neurons also had changes in projections between the *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ spinal cords. Descending Non-Ventral projections increase in the *Sim1* knockout, while ascending Non-Ventral projections decreased, observed in both the total counts (C) and distance plots (D) (*Sim1*$^{+/cre}$, n=5; *Sim1*$^{tLacZ/cre}$, n=3; $p \leq 0.05$).

3.3.3. Ventral V3 interneurons require *Sim1* for proper axon development

The deficiency of V3 projections in *Sim1* null mutants became more obvious and significant at birth (Fig 13-15). As we mentioned earlier, at P0, spinal V3s are predominately commissural. There was no obvious difference with ipsilateral cells (data not shown). As we did with the control animals, we also applied 3kDa dextran at L1 ($Sim1^{+/cre}$, n=4; $Sim1^{tLacZ/cre}$, n=4) and L5 ($Sim1^{+/cre}$, n=4; $Sim1^{tLacZ/cre}$, n=3) of the $Sim1^{tLacZ/cre}$ spinal cords. When summing up the total dextran positive V3 neurons in each spinal cord, we found significant decreases in V3s with descending ($Sim1^{+/cre}$ = 107±10, $Sim1^{tLacZ/cre}$ = 48±7, $p \leq 0.05$) and ascending ($Sim1^{+/cre}$ = 195±14, $Sim1^{tLacZ/cre}$ = 92±9, $p \leq 0.05$) projections to L1, and descending V3 projections to L5 ($Sim1^{+/cre}$ = 100±10, $Sim1^{tLacZ/cre}$ = 51±8, $p \leq 0.05$) (Fig. 13A, B). The only non-significant value was V3s with ascending projections to L5 (Fig. 13B), which were the ascending V3s in the L6/sacral region. To gain a more complete projection profile of mutant V3s at different segments, we further divided the cells into Ventral and Non-Ventral populations and drew the distribution plots related to their projection distance (Fig. 14, 15). Although V3 interneurons in the Lower Thoracic and Higher Lumbar regions of the P0 spinal cord can be separated into 3 subpopulations, this is not the case in the Lower Lumbar and Sacral region, which maintains a 2-population distribution. In addition, as was described above, in $Sim1^{tLacZ/cre}$ spinal cords the Dorsal V3 subpopulation was significantly decreased and it was also difficult to separate dorsal and intermediate V3 interneurons in the mutants. Therefore, we separated V3 interneurons into Ventral or Non-Ventral

populations to compare the axon projection profiles between the *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ V3 interneurons at P0.

With this more detailed analysis, we found that the most profound change in *Sim1*$^{tLacZ/cre}$ spinal cords was a decrease in ascending projection to L1 of Ventral V3s in the lumbar region (Fig. 14A, C). The total number decreased from 56±7 to 14±4 ($p \leq 0.05$). Except the small fraction of the short projection population, there were few ascending Ventral V3s. At the same time, there was also a significant decrease in projecting Ventral V3s in the Lower Thoracic (*Sim1*$^{+/cre}$ = 98±10, *Sim1*$^{tLacZ/cre}$ = 38±6, $p \leq 0.05$) and Lumbar (*Sim1*$^{+/cre}$ = 83±10, *Sim1*$^{tLacZ/cre}$ = 30±4, $p \leq 0.05$) (Fig. 14B, D) regions with descending axons, and V3s in the L6/Sacral region with ascending axons (*Sim1*$^{+/cre}$ = 45±7, *Sim1*$^{tLacZ/cre}$ = 29±6, $p \leq 0.05$). There is also a significant decrease in the number of ascending Non-Ventral V3s in the Lumbar region (*Sim1*$^{+/cre}$ = 139±12, *Sim1*$^{tLacZ/cre}$ = 78±9, $p \leq 0.05$; Fig. 15A, C).

Taken as a whole, our findings show that *Sim1* is required for two post-mitotic processes of the V3 interneurons. Dorsal V3s require *Sim1* to successfully migrate into the dorsal horn, as shown by comparative migration images and subpopulation cell counts. Ventral V3 neurons, as well as possibly some Non-Ventral ones, require *Sim1* to correctly grow commissural axons, as shown in our analysis of ascending and descending projecting neurons in the E14.5 and P0 spinal cord.

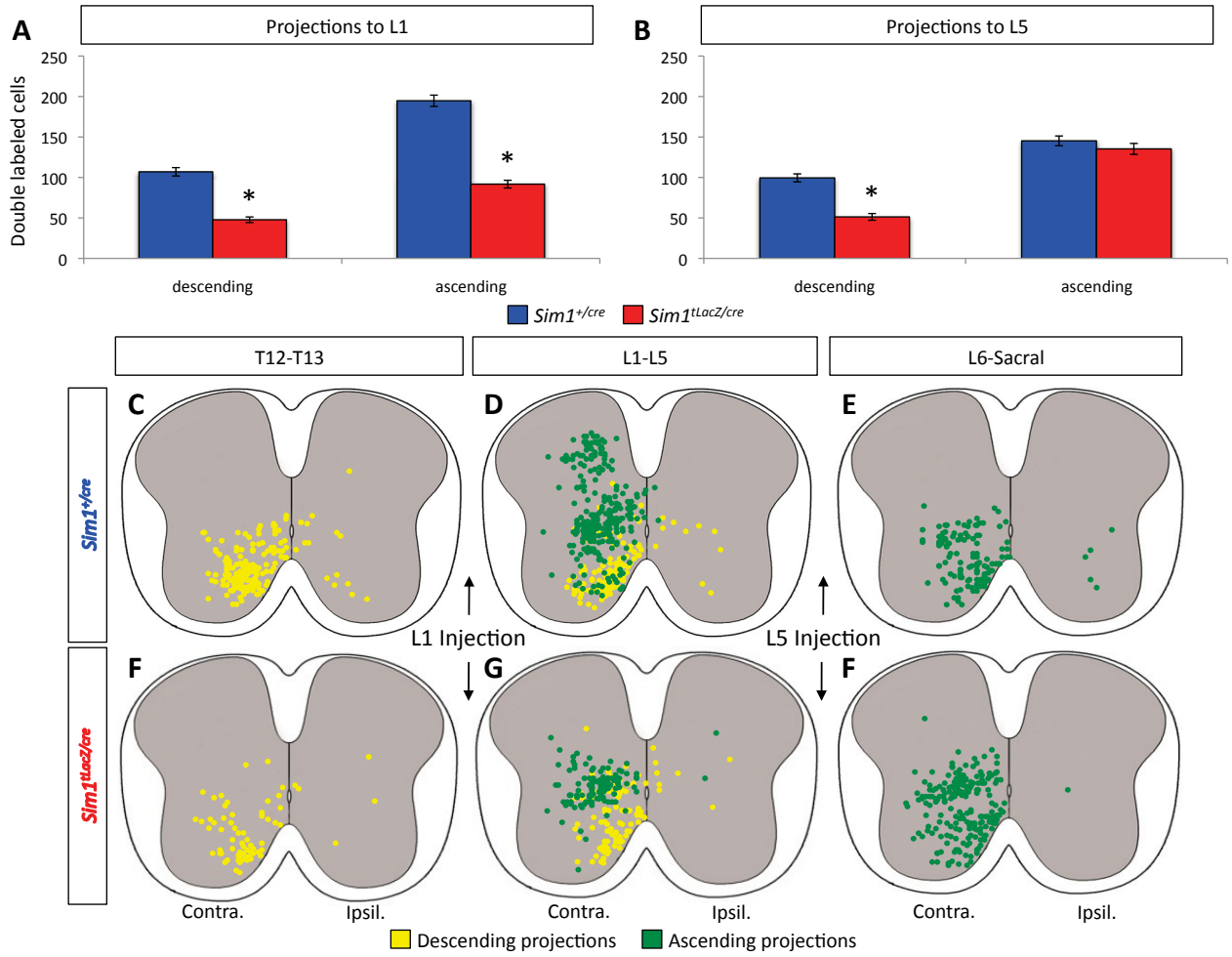
A
Projections to L1
Double labeled cells
250
200
150
100
50
0
*
*
descending
ascending
B
Projections to L5
250
200
150
100
50
0
*
descending
ascending
Sim1+/cre
Sim1tLacZ/cre
T12-T13
L1-L5
L6-Sacral
C
D
E
Sim1+/cre
L1 Injection
L5 Injection
F
G
F
Sim1tLacZ/cre
Contra.
Ipsil.
Contra.
Ipsil.
Contra.
Ipsil.
Descending projections
Ascending projections

Figure 13. *Sim1*$^{tLacZ/cre}$ spinal cords show decreased ascending and descending commissural axon projections at P0.

A, B. Quantification of total ascending and descending axon projections of V3 interneurons to L1 and L5 injection sites in *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ spinal cords. *Sim1*$^{tLacZ/cre}$ spinal cords show significant decreases in both ascending and descending projections to L1 (A) (*Sim1*$^{+/cre}$, n=4; *Sim1*$^{tLacZ/cre}$, n=4; $p \leq 0.05$). *Sim1*$^{tLacZ/cre}$ spinal cords show decreased descending axon projections to L5 (B) (*Sim1*$^{+/cre}$, n=4; *Sim1*$^{tLacZ/cre}$, n=3; $p \leq 0.05$).
C – F. Projection map illustrating the ventro-dorsal soma position of V3 neurons in the *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ spinal cords in the Lower Thoracic region (T12-T13; C, F), Lumbar region (L1-L5; D, G), and L6/Sacral region (E, F) with projections to either the L1 or L5 injection sites. Projection deficiencies in the *Sim1*$^{tLacZ/cre}$ spinal cord appear to be located to the Ventral subpopulation for both ascending and descending projections (compare C and F, D and G), with possible projection deficiencies in the Dorsal subpopulation (compare D and G). Diagrams are composites of 30 μm sections of a single representative spinal cord.

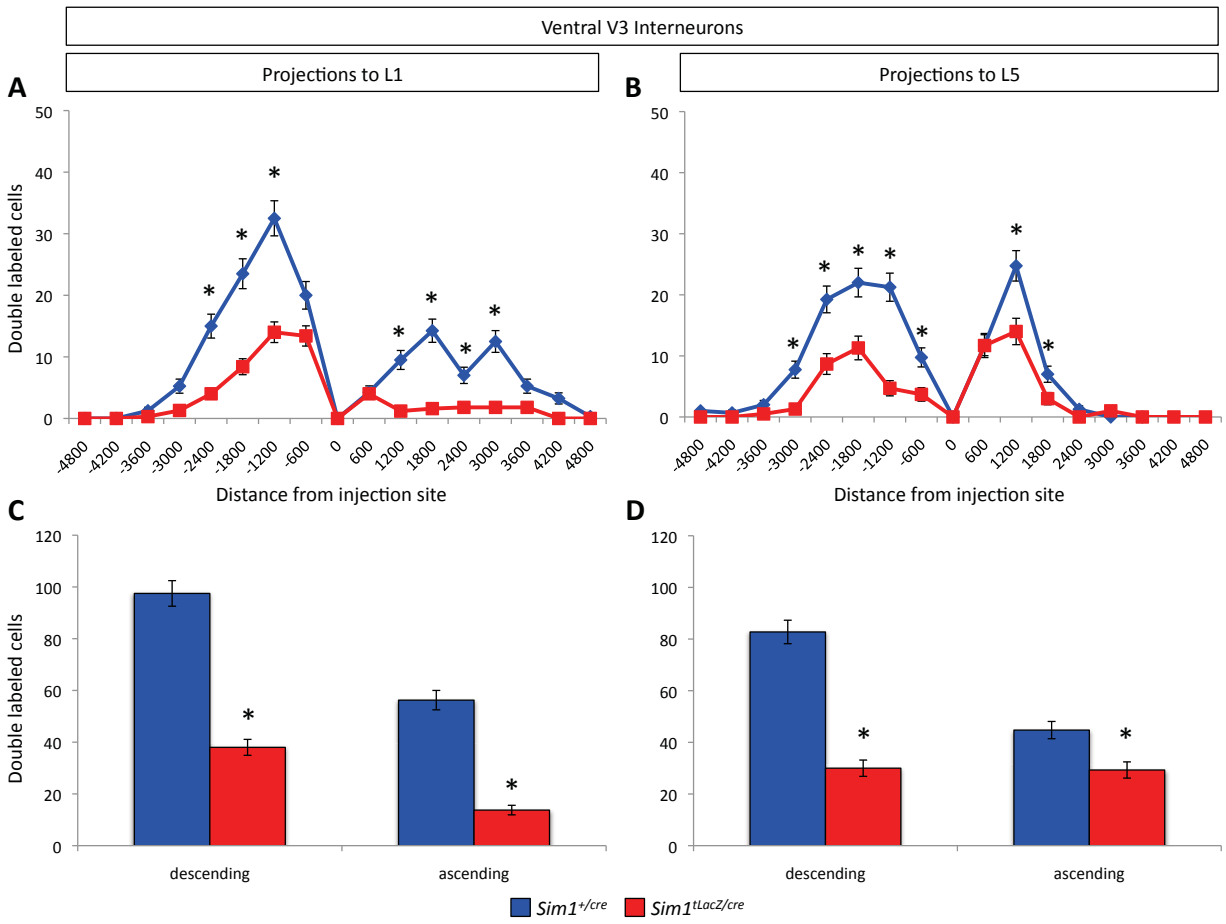

Figure 14. *Sim1*$^{tLacZ/cre}$ P0 spinal cords show a decrease in both ascending and descending axon projections of the Ventral subpopulation.

A, B. Distance analysis of Ventral V3 projections to the L1 and L5 dextran injection site. Ventral V3 neurons show a decrease in the number of biotin-labeled V3 neurons with descending and ascending projections to the L1 injection site (A) (*Sim1*$^{+/cre}$, n=4; *Sim1*$^{tLacZ/cre}$, n=4), and with descending and ascending projections to the L5 injection site (B) (*Sim1*$^{+/cre}$, n=4; *Sim1*$^{tLacZ/cre}$, n=3; $p \leq 0.05$).
C, D. The projection deficiency of Ventral V3 neurons in the P0 spinal cord are summarized, illustrating the deficiency in ascending and descending projections to L1 (C; $p \leq 0.05$) and L5 (D; $p \leq 0.05$).

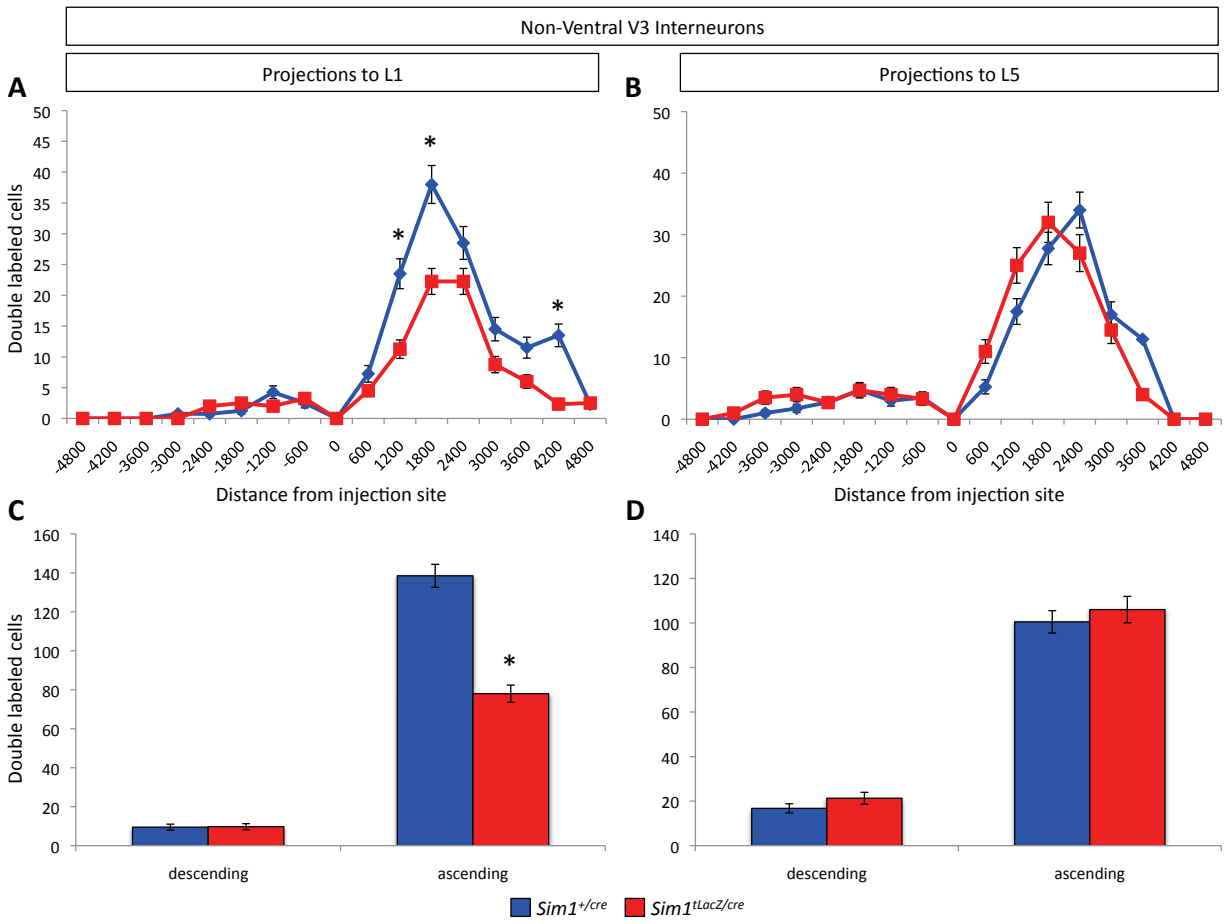

Figure 15. *Sim1*$^{tLacZ/cre}$ P0 spinal cords only show a significant decrease in ascending axon projections to L1 of the Non-Ventral subpopulation.

A, B. Distance analysis of Non-Ventral V3 projections to the L1 and L5 dextran injection site. Non-Ventral V3 neurons show a decrease in the number of biotin-labeled V3 neurons with ascending projections to the L1 injection site (A) (*Sim1*$^{+/cre}$, n=4; *Sim1*$^{tLacZ/cre}$, n=4; $p \leq 0.05$). There is no change in either the ascending or descending projection of Non-Ventral V3 neurons to the L5 injection site (B) (*Sim1*$^{+/cre}$, n=4; *Sim1*$^{tLacZ/cre}$, n=3).
C, D. The projection decrease of Non-Ventral V3 neurons in the P0 spinal cord are summarized, illustrating a decrease in ascending projections to L1 (C; $p \leq 0.05$). No change is noticed in ascending or descending projections to L5 (D).

Chapter 4. Discussion

The molecular mechanisms that generate interneuron diversity in the spinal cord have been an area of intense research for over two decades. Greater understanding of the developmental processes of the interneuron populations that constitute the lumbar spinal cord has provided insights into the organization and functioning of the lumbar Central Pattern Generator.

In this study, we have utilized a transgenic mouse, *Sim1Cre::tdTom,* to characterize the migratory route and the progression of axonal projections of V3 interneurons within the developing mouse spinal cord. We show that the migratory route taken by developing V3 neurons is via a unique dorsolateral migration stream that is unusual for spinal interneurons. We also show that a population of V3 neurons has a transient ipsilateral axon projection at embryonic stages, which become sparse in the newborn mouse.

In a second study, the role of the transcription factor Sim1 is analyzed using a *Sim1* null mutant mouse at different developmental stages. We show that Sim1 plays an integral role in establishing post-mitotic characteristics of the V3 interneuron including their migration and axon projection in lumbar spinal cord.

4.1. V3 neurons show a unique dorsolateral migration pathway to settle into 3 subpopulations

It has been well established that V3 neurons emerge from the ventral-most p3 progenitor domain of the developing neural tube between E9.5 to E11.5 (Jessel, 2000, Zhang et al., 2008). Previous work by Zhang et al (2008) illustrated that V3 neurons show a broad distribution in the mature mouse spinal cord, and are involved in establishing a coordinated and robust motor output from the lumbar central pattern generator. While the function of the V3 population as a whole has been studied, there has yet to be any research into the presence of anatomically or functionally distinct subpopulations.

Our results have shown that starting from the most ventral domain of the neural tube, V3 neurons migrate into different positions along the ventro-dorsal axis of the spinal cord. As early as E12.5 a separation in migratory streams is observed, indicating either a medial or lateral path. Large changes seen between E13.5 to E15.5 show further separation between groups of V3 neurons. A ventral population does not migrate dorsally, and resides in the ventromedial area of Lamina VIII in the P0 spinal cord. The large dorsally migrating population separates into two clusters in the higher lumbar region at E14.5. These two dorsally migrating groups eventually form the Intermediate population within Lamina VII, and the Dorsal population located in the dorsal horn (Lamina IV/V).

The developmental process and distribution of V3 neurons in the newborn spinal cord indicate three anatomically distinct subpopulations. The location of the Ventral subpopulation of V3 interneurons suggests that they may be directly involved in the locomotor CPG. Lesion studies in the neonatal rat have shown that generation of a coordinated left/right alternation depends on neurons in the ventral third of the spinal cord (Kjaerulff and Kiehn, 1996, 1997). Evidence suggesting that the Ventral V3 subpopulation synapses directly onto contralateral motor neurons (Zhang et al., 2008) implicates them in the electrophysiological and functional disruption in motor output observed when the entire V3 population is silenced.

The location of the Dorsal subpopulation of V3 interneurons puts them in the termination field of vGluT1-expressing peripheral afferent fibers (data not shown). This finding, along with the characteristics of sensory interneurons present within the dorsal horn of the spinal cord, suggests that this subpopulation is involved in the relay of sensory information from the periphery to other neurons in the spinal cord and brain structures. The Intermediate subpopulation, present in Lamina VII, is likely involved in both sensory and motor-related functions.

The ventra-dorsal tangential migration of V3 INs is different from dorsal commissural neurons and most other spinal interneurons (Moran-Rivard et al., 2001; Saueressig et al., 1999; Lundfald et al., 2007), which predominately show a dorsal to ventral path. This indicates potentially different mechanisms underlying the migration of V3 INs. Another group of spinal neurons that migrates dorsally

during postmitotic development is the sympathetic preganglionic neurons (SPN). Reelin related pathways have been indicated to play important roles in their dorsal migration (Yip et al., 2009). Previous study also implied that PlexinC-1 might be involved in the migration process of Sim1 positive neurons in the hypothalamus (Xu et al., 2007). However, the molecular pathways guiding the migration process of V3 neurons need to be further investigated.

Another possible explanation for the appearance of distinct subpopulations of tdTom$^+$ neurons in the deep dorsal horn is that not all of them emerge from the p3 progenitor domain, and are therefore not V3 interneurons. A dorsally located progenitor domain may produce neurons that begin expressing *Sim1* at a later stage than V3, which would explain the presence of dorsal populations at stages during development, and in the post-natal spinal cord. Although our analysis on the distribution pattern of tdTom positive cells in the spinal cord at different embryonic stages is consistent with the ventral-dorsal tangential migration of these cells, we cannot rule out the possibility that they may originate from different progenitor domains. More studies into the migratory route of V3 neurons would be required to differentiate these two possibilities.

4.2. V3 neurons show an embryonic switch in their axon projection phenotype

It has previously been suggested that V3 neurons are predominately commissural (85% - unpublished findings, Eric Geiman), however neither the development of

these projections, nor a full classification of the mature V3 projection profile had been done.

A surprising finding during analysis of the E12.5 spinal cord was the presence of an ipsilaterally projecting V3 subpopulation with descending projections. This subpopulation is substantial, with axon lengths similar to those V3 neurons projecting commissurally. While Nissen et al. (2005) found descending ipsilateral projections (dIINs) tend to be positioned more laterally to ascending commissural projections (aCINs), our findings show that the Ventral population of V3 neurons at this time point are intermingled when mapped on a cross section. Even at this early stage, similar to the migration analysis, V3 neurons can be anatomically separated based on their direction of initial projections, as well as their ipsilateral or contralateral phenotype.

The descending ipsilateral projections continue in the E14.5 spinal cord; however, this subpopulation of V3 neurons is no longer the only group with descending projection. At this developmental stage, a third population of V3 neurons arises, with descending commissural projections. It can be suggested that this developmental profile represents 3 subpopulations of V3 interneurons, separated based on their timing and direction of axon projections. A second possibility is that the descending projections arise from a bifurcation of the initial ipsilateral projections. These neurons may temporarily have both an ipsilateral and contralateral component to their axon, both of which are descending.

The transient presence of ipsilateral projections in a commissural interneuron population is a novel characteristic in lumbar interneuron development. The role of this type of projection is unclear. Whether these projections are involved in initiating developmental processes, such as assisting in the dorsal migration of some V3 neurons, more work needs to be done to understand this phenotype. The only other known embryonic change in spinal cord axon projections is observed in V2a interneurons, which initially project both rostrally and caudally, but lose their rostral projections at P0 (Lundfald et al., 2007). Both projections, however, are always ipsilateral. The role of these embryonic V2a rostral projections has not been determined.

4.3. V3 neurons have both ascending and descending multi-segment, commissural axons in the P0 spinal cord

V3 neurons have been shown to be a population of predominately commissural, glutamatergic interneurons (Zhang et al., 2008); however, their full axon projection profile in the post-natal mouse had not been established. In this study, retrograde tract tracing was used to understand the extent of V3 communication between the L1 and L5 regions of the spinal cord. These two regions are presumed to be involved in innervation of flexor (L1) and extensor (L5) muscles of the hind limbs. The communication between these spinal cord levels, mediated through

interneurons, is integral in establishing coordination between antagonistic muscles of the hindlimb – an important aspect of stable locomotion.

Inter-lumbar connectivity between L1 and L5 is seen in the projection profile of the Ventral V3 subpopulation. Neurons in this region (lamina VIII) project both rostrally and caudally, and synapse directly onto motor neurons. Although it cannot be established that double-labeled neurons at each level synapse at the site of dextran-amine injection, it can be suggested that projecting neurons do synapse at or near motor neurons in these spinal levels. The L1/L5 connectivity of ventral V3 neurons, as well as their excitatory phenotype, suggests that they are involved in activation of contralateral antagonistic muscles, which would be required for proper coordination of hindlimb movement during locomotion. This conclusion is supported by the comparison between Netrin-1 and DCC knockout mice. Netrin-1 knockout mice have V3 neurons as the sole commissural population – other commissural populations do not cross the midline (Rabe et al., 2009). These mice exhibit a hopping phenotype. DCC knockout mice, however, have no commissural populations, and have a completely uncoordinated hindlimb pattern (Rabe Bernhardt et al., 2012). These observations support a role for V3 neurons (likely the Ventral subpopulation) in coordinating contralateral antagonistic muscles.

The Ventral subpopulation of V3 is also observed to be the predominate projection from the Low Thoracic region to the L1 spinal segment. These neurons may be involved in relaying body position of the midsection to motor neurons innervating

corrective muscles in the hindlimbs. Due to limitations of dextran-amine tract tracing protocol it could not be established whether Ventral V3 neurons project from the Low Thoracic region to the L5 segment.

Although caudal projections to both L1 and L5 spinal segments are dominated by the Ventral subpopulation, there are small numbers of Intermediate V3 neurons projecting this direction. Interneurons within Lamina VII have been known to be among a bifurcating population (Nissen et al., 2005). The role of the Intermediate V3s is difficult to propose based solely on its location and axon projection profile.

4.4. Sim1 plays a role in post-mitotic development of the Dorsal V3 subpopulation

Sim1 has been implicated to play a role in both development of axon projections in the mammillary bodies (Marion et al., 2005) and migration of neurons in the PVN and SON of the hypothalamus (Xu et al., 2007). Our analysis of the ventro-dorsal position of V3 neurons in the mouse spinal cord at E14.5 and P0 in both *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ reveal the important role of *Sim1* in the migration and axon outgrowth in the spinal cord as well.

Our data have shown that in *Sim1* knockout, V3 neurons are still generated, but there is a lack of a dorsal subpopulation observed in the Lower Thoracic and Higher Lumbar regions of the *Sim1*$^{tLacZ/cre}$ E14.5 spinal cord.

Quantification through cell counts of V3 neurons present within each subpopulation indicates that the dorsal subpopulation is unable to adequately reach the deep dorsal horn, and a resulting increase in the number of V3 neurons in the intermediate subpopulation is observed. The partial migration of prospective Dorsal V3s, and subsequent "stalling" in the intermediate region, suggests that V3 migration may be due to more than one process and gradient sensitivity. Sim1 may be required for the second stage of dorsal migration forming the intermediate and dorsal subpopulations. Dorsal interneurons that fail to reach their dorsal destination may lose their ability to properly receive peripheral inputs, and may therefore no longer be involved in the sensorimotor pathway. A better understanding of the impact of this migration deficit may be observed by whole-cord electrophyiological analysis of the *Sim1*$^{tLacZ/cre}$ spinal cord, with stimulation via the dorsal roots. As we pointed out earlier, this lack of dorsal tdTom positive neurons may also be due to the defect of Sim1-expressing cells from another progenitor domain.

An analysis of the total V3 neurons present within the *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ spinal cords reveals an interesting, although complicated change in the Sim1 mutants. *Sim1*$^{tLacZ/cre}$ spinal cords show consistently higher numbers of V3 neurons, with significant differences noted in the Lower Thoracic region, as well as L3 and L4 at P0. Such increase is consistent with the finding that *Sim1*$^{tLacZ/cre}$ neurons undergo

less programmed cell death than the *Sim1*$^{+/cre}$ counterparts in the hypothalamus (Xu et al., 2007).

4.5. Sim1 is required for proper axon development of Ventral and Non-Ventral V3 subpopulations

The dextran tracing experiments were performed to determine the projection pattern of V3 neurons in control and Sim1 knockout mice. These studies revealed significant decreases, but not absence, of V3 commissural projections, especially in the Ventral subpopulation.

Analysis of the E14.5 and P0 spinal cord revealed no change in ipsilateral projections in either ascending or descending V3 axons between *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ spinal cords. This suggests that mutant *Sim1* neurons might not simply change from contralateral to ipsilateral projections. We also didn't observe any dramatic difference in width or intensity of V3 axons at the ventral commissure of E14.5 cords, suggesting that in *Sim1*$^{tLacZ/cre}$ cords, the deficiency in the outgrowth of V3 neurons may occur after crossing the midline. Using our tracing method, however, we were not able to catch all of the short (inter-segmental) projecting axons. A more detailed analysis of these short projections may shed more light on this issue.

The role of *Sim1* in correct axon guidance has been noted previously in mammillary body projections to both the thalamus (Mammillothalamic tract – MTT) and the tegmentum (Mammillotegmental tract – MTEG) (Marion et al., 2005). This study implicated Sim1 (and Sim2) in correct axon guidance and fasciculation during mammillary body development. An important difference between this study and our own is that the MTT and MTEG are ipsilateral projections that show a deviant midline projection when Sim1 is knocked out. Marion et al. (2005) found that Sim1 downregulates Rig-1/ROBO3 expression, causing high levels of ROBO-1 to be expressed in the growth cone, and an ipsilateral phenotype to be maintained. This mechanism cannot be applied to the role of Sim1 in V3 spinal interneurons because of its commissural phenotype. The role of Sim1 in V3 spinal interneurons is unclear. It may have an opposite effect on Rig-1/ROBO-3 expression, acting to upregulate it. This would decrease the sensitivity of developing V3 axons to Slit repulsion, allowing axons to cross the midline. In the case of $Sim1^{tLacZ/cre}$, sensitivity to Slit may be maintained and V3 axons cannot successfully cross the ventral commissure, and therefore not develop correctly. Further analysis would be required in order to confirm this connection.

Commissural axons that have crossed the midline are sensitive to two known chemical gradients. The Wnt gradient is high rostrally and can act as an attractive cue via the Frizzled (Frz) receptor (Lyuksyutova et al., 2003) or a repulsive cue via the Ryk receptor (Lu et al., 2004; Liu et al., 2005). The Shh gradient is high caudally, and acts as a repulsive cue to many developing axons (Bourikas et al., 2005). The

Ventral V3 subpopulation in *Sim1*$^{tLacZ/cre}$ mice shows the strongest defect in axon guidance. If the deficiency is in post-crossing sensitivity it can be suggested that Ventral V3 neurons respond to a different cue, or through a different receptor, than the most Non-Ventral neurons. There is, however, a small subset of Non-Ventral V3 neurons that show a deficiency in ascending projections. These neurons may be sensitive to the same cues as the Ventral neurons.

While the migration and axon deficiencies observed in the *Sim1*$^{tLacZ/cre}$ spinal cord are significant, they are not a complete elimination. Some V3s are still able to reach the dorsal horn to become dorsal V3s, and some Ventral V3s are still able to project properly. The role of Sim2 therefore must be considered when analyzing this data. Sim2 has been shown to compensate, to an extent, for the absence of Sim1 in the development of axon projections of the MTT and MTEG (Marion et al., 2005). It could be considered that Sim2 is upregulated in the absence of Sim1 in V3 neurons, attributing to the significant, although somewhat subtle, phenotype observed in the *Sim1*$^{tLacZ/Cre}$ spinal cord.

4.6. Future Directions

Understanding the expression timeline of *Sim1* in each subpopulation of V3s would provide information as to differential use of this transcription factor at different times. For example, *Sim1* may be expressed for a longer period of time in dorsally migrating V3s compared with Ventral ones. *In situ* analysis of the developing spinal

cord would provide this information. *In situ* hybridization could also be used to determine if Sim2 is used differently in V3 subpopulations, or possibly upregulated in *Sim1*$^{tLacZ/cre}$ spinal cords.

An important next step in understanding the subpopulations of V3 interneurons would be to perform a full analysis of other transcription factors that are upregulated or downregulated between the *Sim1*$^{+/cre}$ and *Sim1*$^{tLacZ/cre}$ neurons. Identification of a transcription factor that is expressed in one subpopulation and not the other would lead to exciting genetic analysis of this subpopulation, and further our understanding of the diverse population of V3 interneurons.

4.7. Conclusion

The goal of our study was two-fold: separate the V3 population into anatomically distinct subpopulation, and understand the role of the transcription factor *Sim1* in V3 development.

We observed that V3 neurons settled in distinct subpopulations following migration – Ventral, Intermediate, or Dorsal. These subpopulations were also distinct in their axon projection profile. Ventral V3s contained both ascending and descending projections, Intermediate V3s were predominately ascending but contained a few descending, and Dorsal V3s were exclusively ascending.

Through our analysis of the *Sim1* knockout spinal cord, we determined two distinct roles that *Sim1* plays in V3 development. This transcription factor is involved in both proper migration and axon projection of V3 interneurons. Interestingly, these roles were subpopulation-specific, providing not only an anatomical separation of V3 neurons, but a genetic separation as well. Dorsal V3s require *Sim1* for proper dorsal migration, while Ventral and select Non-Ventral V3s require *Sim1* for proper axon development.

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