Connectivity of dI3 Interneurons in the development of mice spinal cord

Carl Farah

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Department of Science
Faculty of Biology
University of Ottawa

Abstract

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By

Carl Farah

Maturation of motor control, including movements that can be autonomously generated by spinal circuits, relies on the development of key inputs to spinal circuitry. In particular, the development of supraspinal, sensory and motor fibers come together to form organized spinal circuits capable of producing skilled movements that are volitionally controlled. Primitive reflexes such as the palmar grasp reflex (PGR) are known to disappear during development; presumably giving way to more volitional control of hand grasping. However, the underlying changes to the spinal circuitry responsible for this transition remain to be determined. dI3 INs, a class of dorsal spinal interneurons, have positioned themselves as key mediators of reflexive grasping in early development and grasping in adult mice. The first aim of the study focused on determining the developmental time point at which the PGR disappeared. Our studies demonstrated that the PGR was lost by the third week of development. The second aim of this study focused on identifying changes in sensory innervation, presynaptic inhibition and supraspinal excitation to dI3 INs that might account for the loss of this reflex. Our studies demonstrated that while sensory innervation remained constant during development, presynaptic inhibitory terminals onto sensory afferents were found to increase during development. In addition, we report that dI3 INs receive decreasing corticospinal (CST) input during development. While these developmental changes do not fully account for the disappearance of the PGR, they provide valuable insights into how a reflex centered on a particular population develops.

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CHAPTER 1
General Introduction

1.1 Overview

The spinal cord is the essential link between the brain and body. However, the spinal cord is much more than a simple information highway which passively relays signals. Rather, the spinal cord should be regarded as a highly active member of the central nervous system, capable of shaping signals and in certain situations even being able to coordinate its own output without intervention from the brain. Altogether, the spinal cord fulfills two essential functions; first, as a processing center for incoming sensory information and second, as a controller for motor and reflexive actions.

These functions are implemented by the spinal cord at all areas of the body below the head. As such, the spinal cord can be divided into four regions (from rostral to caudal): the cervical, thoracic, lumbar and sacral regions. Each region being composed of multiple segments at which spinal nerves emerge from both sides of the spinal cord and innervate specific areas of the body. These spinal nerves carry motor, sensory and autonomic signals to and from the body (Kandel et al. 2013). Visualizing a transverse section of the spinal cord reveals the trademark butterfly shape of the grey matter. The distribution of cells within the gray matter is grouped into divisions called laminae; each lamina (I-X) unites cells based upon their cytoarchitecture.

1.2 The spinal cord: function and roles

1.2.1 Sensory processing

As previously mentioned, the spinal cord is a major entry point for sensory information. Sensory information is relayed to the spinal cord through sensory neurons located in dorsal root

ganglions (DRGs) whose cells bodies lie in close proximity to the spinal cord. DRG nerve terminals in peripheral areas such as the skin, muscles, and viscera are equipped with diverse receptors capable of sensing distinct sensory modalities including thermoception (temperature), proprioception (sense of self), mechanoception (touch) and nociception (pain). Cutaneous afferents mostly terminate in the dorsal horn with noxious stimuli (conveyed by Aδ and C fibers) mainly terminating in the superficial laminae (Lamina I-II) whereas non-noxious stimuli (conveyed by Aβ fibers) project to the deeper dorsal horn (laminae V-VII) (Abraira and Ginty 2013). Proprioceptive afferents, in particular, primary muscle afferents (Type I) mainly project to the ventral horn (lamina XI) and to ascending pathways to the brainstem whereas the second subset of proprioceptive afferents (secondary or Type II) terminate in the deep dorsal horn (laminae V-VII). It is also worth noting that a subset of low-sensory mechanoreceptive afferents also project to the brainstem where they are ultimately relayed to areas of the brain such as somatosensory cortex for higher-order functions such as somatosensation. Incoming sensory information often undergoes synaptic integration by spinal neurons upon entering the spinal cord; these signals can subsequently act locally and/or be relayed to supraspinal centers for further processing (Bui et al. 2015).

In addition to affording information about our surroundings, sensory information can be employed to update motor actions in real-time. Perhaps the most studied example being locomotion, where sensory feedback has been implicated in the timing (Pearson et al. 1998), magnitude (Nielsen 2004), and coordination of muscle contractions (Aprigliano et al. 2015). Similar to locomotion, grasping requires constant sensory information such that on-line visual control mediates the scaling of grip aperture as well as hand placement (Volcic and Domini

2016) while cutaneous feedback mediates the strength of grip during hand-held object manipulation (Augurelle 2002). Moreover, proprioceptive information has been demonstrated to be essential for the transport and grasping of objects (Gentilucci et al. 1994). In summary, the spinal cord is central to not only sensory information transmission but to the processing of this information for adjustments in motor control.

1.2.2 Motor output

With regards to the process of motor control, the spinal cord serves as the signal processor between the brain where most motor commands originate, and the muscles, which are the actuators of motor activity. More specifically, the spinal cord contains the neural circuitry required to translate motor commands into coordinated muscle activation thus leading to movement. Initiation of movement occurs in the motor cortex and is relayed to the spinal cord through a multitude of descending tracts originating from the brain (e.g. corticospinal tracts) or the brainstem (e.g. reticulospinal, vestibulospinal or rubrospinal tracts) (Rothwell 2012). These descending tracts either directly or by way of spinal interneurons (INs) contact motoneurons (MNs) who are themselves in command of bringing life to the complex patterns of movements. In addition, the spinal cord contains many neural circuits that can autonomously coordinate complex movement such as the spinal circuits essential for locomotion (Frigon 2012).

In contrast to coordinated movements, reflexes are often thought of as involuntary patterns of motor responses brought about by the presence of a sensory stimulus. The neural correlate of a reflex is referred to as a reflex arc and is composed of an afferent (sensory) and efferent (motor) signal, often times connected by one or more interneurons. It is worth noting that certain reflexes make use of supraspinal pathways whereas others are entirely confined to

the spinal cord. Reflexes that develop very early in fetal development and that disappear through maturation are called primitive reflexes. These primitive reflexes include the grasp, moro, and rooting reflex among others and are believed to be a set of evolutionarily conserved behaviours that promote survival of neonates. The neural circuitry of these reflexes does not disappear but is believed to instead fall under the control of higher centers where it can be recruited for voluntary control. It is interesting to note that primitive reflexes re-appear in patients with frontal cortical lesions, solidifying the theory that these reflexes are under cortical control (Futagi et al. 2012). While reflexes are often discussed within the context of neurological testing, their role in ensuring proper motor control is not yet fully understood.

As described above, the diverse general roles of the spinal cord are well identified; however, the specific contribution of spinal neuron populations remains elusive. The exercise of identifying spinal neuron populations remains very fluid as subsets of these population are still being described based on various types of criteria such as location, connectivity, and molecular profile to just name a few (Bikoff et al. 2016). Indeed, being able to correctly identify spinal neuron populations is only the first step in characterization; the second and arguably more useful for understanding spinal cord function is the ability to properly associate specific functions to each population and or subsets of populations. An approach that has made great progress faced with this challenge is the organization of spinal neurons based upon their developmental origin. I will use this approach in describing the classification of spinal neurons.

1.3 Development of spinal neurons

Spinal neuron development is known to arise through a combinatorial code of transcription factors. Early in embryonic development and during the process of neurogenesis,

gradients of diffusible morphogens such as Sonic hedgehog (Shh) are released from the ventral floor plate and the dorsal roof plate and act on neural progenitors to activate various transcription factors. Cross-repressive and permissive interactions amongst transcription factors lead to the emergence of thirteen progenitor pools of spinal neurons that express different transcription factors depending on their positioning in these morphogen gradients (Roelink et al. 1994). These progenitor domains, depending on the expression of further postmitotic transcription factors, mature into over 20 types (and counting) of functionally distinct interneurons in addition to MNs (Lu et al. 2015).

These newly differentiated interneurons can then be divided into two groups, the ventral (V0-V3) interneurons arise from progenitors found in the ventral horn and are generally associated with motor function whereas the dorsal interneurons (dI1-dI6) arise from progenitors in the dorsal horn and are predominantly associated with sensory processing (Vallstedt and Kullander 2013; Francius et al. 2015). An example of one of these interneurons domains is the V0 IN population, which is distinguished by the expression of the transcription factor Dbx1, which is involved in mediating left-right alternation at different speeds (Talpalar et al. 2013). Specifically, the V0 IN population is divided into inhibitory $V0_V$ (ventral) INs that mediate left-right alternations at low speeds and excitatory $V0_D$ (dorsal) INs that mediate left-right alternation at high speeds. In addition, there exists a third subpopulation, the cholinergic $V0_C$ INs, for which a functional role has yet to be ascribed (Siembab et al. 2010). It is worth noting that many of the functionally distinct population of interneurons can be further divided into functionally distinct subpopulations (Jankowska and Steve 2011; Bikoff et al. 2016).

1.3.1 Dorsal interneurons 3

Dorsal interneurons can be further divided into two subclasses; those lacking Lbx1 expression are denoted as Class A (dI1-3) whereas those expressing Lbx1 are denoted as Class B (dI4-6) (Lu et al. 2015). Dorsal interneurons 3 (dI3 INs) are characterized by their expression of Brn3a, Tlx3, and Drg11 amongst other transcription factors (Mizuguchi et al. 2006; Zou et al. 2012; Lu et al. 2015). More importantly, they were further characterized by their expression of Isl1, a LIM homeodomain transcription factor also expressed in MNs but exclusively found in dI3 INs amongst neurons of the dorsal horn (Pfaff et al. 1996; Liem et al. 1997). dI3 INs were mainly located in lamina V-VII at similar densities in the cervical and lumbar segments (Figure 1) (Bui et al. 2013). In-situ hybridization for vesicular transporter 2 (vGLUT2) mRNA and single cell labeling have determined that dI3 INs are predominately glutamatergic with ipsilateral projections to MNs. Further, dI3 INs were found to be contacted by primary sensory afferents as demonstrated by vGLUT1 immunohistochemical labeling. Electrophysiological experiments confirmed that dI3 INs received monosynaptic input from proprioceptive and low-threshold cutaneous afferents (Bui et al. 2013). The majority of these vGLUT1 contacts were characterized by an absence of parvalbumin, suggesting that sensory information is most likely transmitted from cutaneous as opposed to proprioceptive afferents (Bui et al. 2013). Recent tracing experiments have determined that dI3 INs project to the lateral reticular nucleus (LRN) (Pivetta et al. 2014), a pre-cerebellar nucleus involved in posture and paw placement (Santarcangelo et al. 1981). In addition, further immunohistochemical labeling determined that dI3 INs are contacted by considerable amounts of vGLUT2 terminals (Bui et al. 2013). The origin of these excitatory connections is not yet known but most likely encompasses inputs from spinal interneurons as well as from supraspinal centers.

Transgenic mice named dI3^{OFF} in which dI3 IN neurotransmission was exclusively genetically silenced through conditional ablation of vGLUT2 in dI3 INs, were employed to further tease apart the role of dI3 INs (Bui et al. 2013). In control mice, in vitro stimulation of the sural nerve, which predominantly carries cutaneous information, generated a robust di-synaptic motor response. Interestingly enough, this reflex was completely absent in dI3^{OFF} mutants, suggesting that dI3 INs are indeed implicated in a disynaptic microcircuit connecting cutaneous afferents with MNs (Bui et al. 2013). Additional experiments suggest that dI3 INs can excite spinal locomotor networks (Bui et al. 2016). In turn, they receive rhythmic inhibition from spinal locomotor networks that is in phase with the swing phase of locomotor activity. How this circuitry influences locomotor activity is not yet fully understood. Experiments with dI3^{OFF} mice suggest that during treadmill locomotion, dI3 INs play an accessory role to the kinematics of locomotion. However, these neurons may be important for providing a drive to spinal locomotor networks to compensate for the loss of descending drive following spinal cord injury. It is important to consider that at early postnatal stages, dI3 INs are capable of driving locomotion; however genetic silencing of these neurons does not disrupt locomotion in adult mice suggesting that their involvement changes during maturation. Perhaps the most striking deficit of dI3^{OFF} mice, however, was an apparent lack of grasping ability when tested with the wire hang test. In addition, mutants showed a decreased incidence of the palmar grasp reflex, further implicating dI3 INs in the mediation of grasping (Bui et al. 2013).

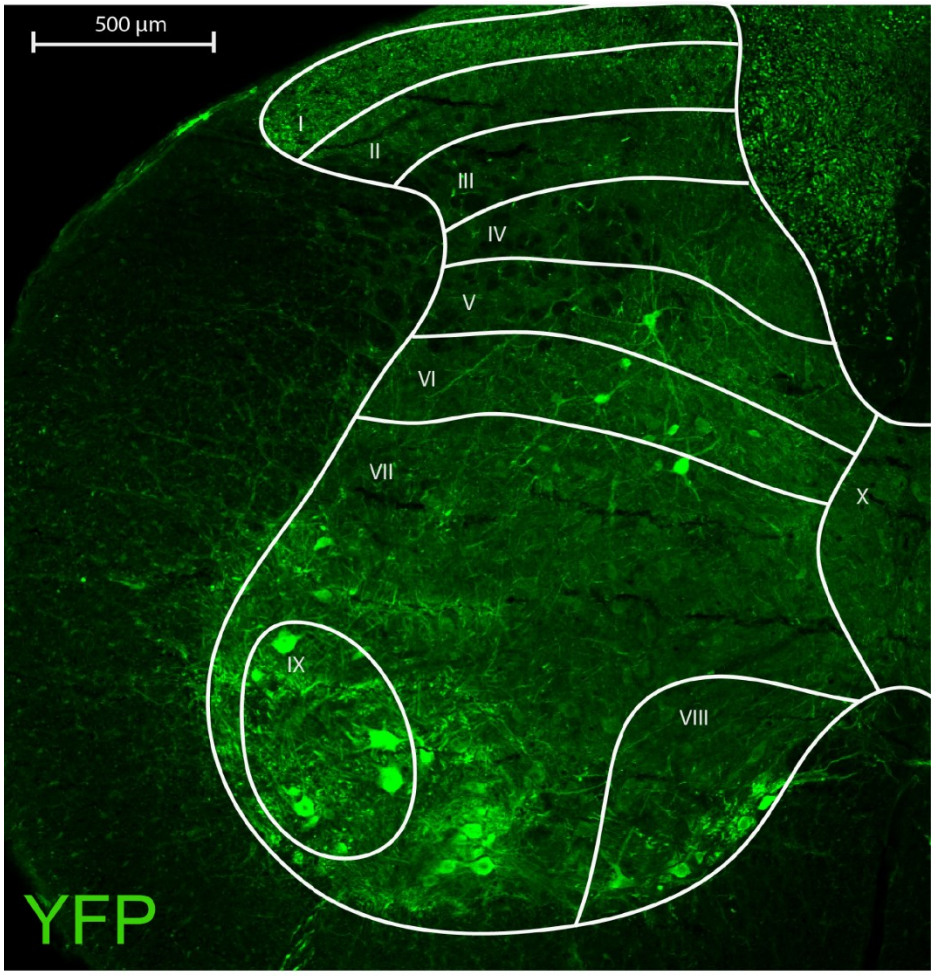

Figure 1. Laminar distribution of cervical dI3 INs.

Confocal fluorescence image of spinal cord from P60 Isl1-Cre; Rosa26-lox-stop-lox-YFP mice. Laminar boundaries are based upon Rexed's classification. Note that motoneurons in lamina IX and in ventromedial aspect of ventral horn are also labelled by YFP. dI3 INs are YFP labelled cells in laminae V-VII.

In brief, it appears that as the nervous system matures there is a corresponding shift in the role of dI3 INs. More specifically, in early development, dI3 INs are found be essential in mediating the palmar grasp reflex whereas at later development time points they are essential in mediating grip strength. In light of this, it is likely that the change in function of dI3 INs reflects changes in circuitry. Indeed, development of the nervous system is associated with many changes in circuitry such as sensory innervation, maturation of descending tracts and much more. Studying the development of dI3 INs presents itself as a unique opportunity to understand how the connectivity of a particular well-defined cell class is correlated with the maturation of well-defined motor behaviours.

1.4 Development of sensory afferents in the spinal cord

Our environments contain large amounts of sensory stimuli which our nervous system must properly process in order to adapt to our changing environment. As such, spinal circuits controlling movements require constant sensory information in order to adapt to continuously changing conditions surroundings or to interact with objects. Removal of cutaneous afferents from the hand limits our ability to skillfully handle objects of different textures (Johansson and Westling 1987). Alternatively, mice devoid of sensory feedback during locomotion demonstrate significant reductions of cross-joint muscle coordination (Akay et al. 2014). There are many more such examples showing how our movements are impaired by lack of sensory information.

Understanding the development of sensory innervation is essential in explaining the maturation of spinal motor circuits. Sensory fibers can be first seen entering the dorsal horn at embryonic day 14.5 (Ozaki and Snider 1997). The first collaterals that enter the dorsal horn are proprioceptive afferents that then migrate ventrally and are afterward followed by cutaneous

afferents that migrate to the dorsal horn (Davis et al. 1989). Similar to the development of spinal interneurons, the development of cutaneous and proprioceptive afferents relies on the specific spatiotemporal expression of transcription factors in spinal and/or sensory neurons. In particular, expression of Erg3 has been found to be essential in the proper development of Ia proprioceptive afferents (Chen et al. 2002). Alternatively, the expression of Drg11 is essential in the proper patterning of cutaneous sensory afferents (Chen et al. 2001).

During the first three postnatal weeks, there is a continual refinement of sensory connections in the spinal cord. As such, in mice, laminar termination of cutaneous afferents within the spinal cord changes considerably after birth (Fitzgerald et al. 1994). In parallel, the receptive fields of cutaneous afferents have been known to decrease during development (Fitzgerald and Jennings 1999). It is not yet known if sensory refinement is implicated in the loss of primitive reflexes, however, the changes in the sensory processing described above support the idea that as the nervous system develops it becomes less reactive in nature (Altman and Bayer 2001). In addition, it is interesting to remark that the mechanical threshold of cutaneous mechanosensitive primary afferents does not change during development (Fitzgerald 1987). Therefore, this suggests that changes in reflex sensitivity most likely arise from changes in signal processing by central mechanisms. In light of this, one might expect to find a reduction of sensory afferents or a gating of sensory information onto dI3 INs as they mature.

Several spinal interneurons integrate sensory information for motor control (Bui et al. 2015), however, to our knowledge, the maturation of sensory afferentiation onto a particular cell class of spinal interneurons has yet to be studied. The study of dI3 INs is poised to offer unique

insights into how sensory maturation affects the functional role of one particular cell class in motor control.

1.5 Development of motor control

As animals develop, reflexive movements mediated by spinal sensorimotor circuits are replaced by purposeful and voluntary movements that result from the concerted activity of networks of neurons in the motor cortex, basal ganglia, cerebellum, and brainstem. These centers, through descending and ascending pathways, constantly communicate with the spinal cord to coordinate motor actions.

Descending motor tracts can be functionally divided into two groups, the pyramidal, and extrapyramidal tracts. The pyramidal tract is responsible for voluntary control of musculature whereas the extrapyramidal tract controls involuntary and automatic control of musculature. The pyramidal tract is composed of both the corticobulbar tract (CBT) and the corticospinal tract (CST). Both these tracts originate in the cortex and consist of upper MNs terminating in the brainstem (CBT) and the spinal cord (CST) (Lemon 2008). Interestingly enough, the CST is thought to be the main descending tract involved in the control of skilled movements (Porter and Lemon 1993). The majority of CST upper MNs synapse on spinal interneurons whereas a small proportion directly synapse onto lower spinal MNs, which in primates is believed to produce fine motor control of the digits (Porter 1985; Bareyre et al. 2005).

The activity of neonates is largely reflexive in nature and this is partly related to the CST not being refined at birth but maturing in the early stages of development (Kudo et al. 1993). Genetic labeling of CST fibers in mice has revealed that cervical and lumbar segments were

reached by postnatal day 1 and 5, respectively (Bareyre et al. 2005). Interestingly enough, developing CST fibers can be extensively found in the ipsilateral gray matter, as opposed to being restricted to the contralateral as one would expect (Qun and Martin 2011). During maturation, the CST undergoes significant synaptic pruning as remarked by the reduction in innervation at later developmental stages (Martin 2005). This pruning is most likely to lead to the contralateral feature of the CST in the adult nervous system. Maturation of frontal brain regions has been associated with the disappearance of primitive reflexes (Isakov et al. 1984; Jolles et al. 1993). It is worth noting that the palmar grasp reflex (PGR) can be consistently elicited from postnatal day 7 onwards and disappears during maturation (Fox 1965).

The extrapyramidal tract is mainly composed of the rubrospinal tract (RBT), the reticulospinal tract (ReST) and the vestibulospinal tract (VST). The VST relays information from the brainstem to the spinal cord and is essential in maintaining head and eye coordination as well as posture. The RBT connects the red nucleus; a midbrain structure involved in motor coordination, to the spinal cord and more particularly to the cervical and lumbar enlargements. The RBT has been known to be involved in shaping hand movements, especially during reaching and grasping movements (van Kan and McCurdy 2001). Lastly, the ReST connects the reticular formation, a group of brainstem nuclei, to the spinal cord. The activity of the ReST during reaching movements has been demonstrated in cats (Schepens and Drew 2006) and primates (Davidson and Buford 2006). Interestingly enough, interneurons involved in hand movement were found to receive convergent input from both the CST and ReST (Riddle and Baker 2010), with some studies placing this convergence at 50% (Baker 2011). As mentioned earlier, the

disappearance of the PGR is most likely associated with maturation of descending tracts; however, the developmental contribution of each of these tracts is not yet well understood.

Descending tracts can be further identified by their neurotransmitter phenotype, with the majority of descending motor tracts such as the CST, VST and RBT being excitatory in nature. The RBT, VST and a portion of the ReST express vGLUT2 while the CST distinguishes itself by vGLUT1 expression, which is also found in primary afferents in the spinal cord. CST axons can be further differentiated from sensory afferents by their expression of protein kinase C gamma (PKCγ) (Finger et al. 2002). It is worth noting that a minority of ReST axons are inhibitory and express the vesicular GABA transporter (vGAT). In light of this, inhibition from higher centers is most likely mediated by glutamatergic activation of inhibitory spinal interneurons instead of stemming from dedicated descending inhibitory tracts.

Development of the CST has the possibility of affecting dI3 INs in at least three ways. First, maturation might lead to an increase in CST terminals onto dI3 INs. Second, maturation might lead to increased CST input onto excitatory spinal neurons contacting dI3 INs. Third; maturation might lead to an increase in CST input onto inhibitory interneurons connected to dI3 INs. It is interesting to note that the latter pathway represents an antagonist mechanism of activation when compared to the others. Indeed, the third hypothesis correlates more favorably with the idea that maturation of dI3 INs is accompanied by reduced excitability. In brief, the proper integration of descending pathways is essential in generating proper motor behaviours.

The study of dI3 INs will offer unique insights about how supraspinal centers integrate signals for voluntary movements and how these circuits change during development. Further, answering these questions will help us understand how a circuit mediating a primitive reflex matures into a circuit essential for grasping.

1.6 Presynaptic inhibition of primary afferents

The skin is the largest sensory organ and receives a constant influx of sensory information that is transmitted to the central nervous system by sensory neurons innervating mechanoreceptors in the skin. Similarly, a number of sensory neurons innervate muscles to detect the length of muscles and the velocity of muscle contraction. As such, the nervous system employs different mechanisms to limit and gate incoming sensory signals. One such gain control system is presynaptic inhibition of primary afferents and is mediated by a subset of GABAergic interneurons in the spinal cord known as GABApre neurons (Eccles et al. 1961, 1962; Burrows and Matheson 1994; Capaday 2002; Rossignol et al. 2006; Betley et al. 2009). Presynaptic inhibition is thought to be recruited by a trisynaptic pathway by which primary afferents synapse onto glutamatergic interneurons that in turn activate GABApre neurons that then form axo-axonic synapses onto primary afferents (Sonner and Ladle 2013). However, recent evidence also supports a more direct disynaptic pathway by which primary afferents directly synapse onto GABApre neurons (Hochman et al. 2010). Presynaptic inhibition can be also be mediated by descending inputs such as the CST that activate GABApre neurons (Canedo 1997; Wall and Lidierth 1997; Rudomin and Schmidt 1999). Importantly, presynaptic terminals are readily distinguishable from other inhibitory terminals by their distinctive expression profiles of GABA synthetic enzymes. In addition to expressing GAD67, GABApre terminals selectively express GAD65 (Soghomonian and Martin 1998; Hughes et al. 2005). As well, GABApre terminals

contact terminals from primary afferents and do not contact the cell body or dendrites of spinal neurons. Presynaptic inhibition has been demonstrated to be essential in executing skilled movements such as reaching and grasping. Indeed, ablation of GABApre neurons resulted in forelimb oscillations during reaching tasks (Fink et al. 2014). Given the importance of presynaptic inhibition, it is possible that primary afferents contacting dI3 INs receive presynaptic inhibition.

Genetic labeling of dI4 INs through their expression of Ptf1a specifies a population of GABAergic interneurons localized in the dorsal horn (Glasgow et al. 2005) that was later revealed to mediate most if not all presynaptic inhibition. Following the development of Ptf1a labeled neurons provides insights into the maturation of presynaptic inhibition in the spinal cord. At birth, cutaneous afferents were found to be contacted by presynaptic terminals but lacked expression of associated synaptic markers (Betley et al. 2009). By postnatal day 7, presynaptic terminals contacting sensory boutons contained high levels of appropriate synaptic markers (Betley et al. 2009). Indeed, the first week of development is required in order for primary afferent stimulation to elicit presynaptic inhibition (Sonner and Ladle 2013). Finally, by postnatal day 15, the vast majority of labeled interneurons contacting sensory terminals expressed appropriate synaptic markers of presynaptic inhibition (Betley et al. 2009).

Recently, the mechanisms by which presynaptic inhibition is regulated have begun to be elucidated. In particular, the release of glutamate from primary afferents has been shown to signal the development of presynaptic inhibition in GABApre neurons (Mende et al. 2016) , linking the activity of sensory afferents with the development of presynaptic inhibition. Further,

immunohistochemical experiments have determined that cutaneous and proprioceptive afferents appear to be contacted by distinct subsets of Ptf1a labeled GABApre neurons. Indeed, presynaptic terminals contacting cutaneous afferents expressed GlyT2, ENK, and NPY whereas none of these markers were present on presynaptic terminals associated with proprioceptive afferents (Betley et al. 2009).

In view of the importance of dI3 INs in the palmar grasp reflex, it is reasonable to posit that changes in presynaptic inhibition of sensory inputs to dI3 INs might mediate the elimination of this primitive reflex during development. Indeed, the study of dI3 INs offers the ability to follow the maturation of presynaptic inhibition and to relate this maturation to the transition from reflexive to voluntary grasping.

1.7 Statement of objectives

Considering that hand control and locomotor control are known to evolve as the motor system develops, we asked how the connectivity of dI3 INs matures (Figure 2). Our goal is to study the connectivity of dI3 INs during the development of mice. To reach this goal**,** Isl1-Cre; Rosa26-YFP transgenic mice were divided into multiple age groups from early development to adulthood. We first determined when the palmar grasp reflex (PGR) disappears during development. Next, using immunohistochemistry, we sought to analyze how the following projections to dI3 INs develop: 1. Sensory inputs to dI3 INs (vGLUT1/GFP). 2. Presynaptic inhibition to dI3 INs (vGLUT1/GFP/GAD65). 3. Corticospinal projections to dI3 INs (vGLUT1/GFP/PKCγ). The results from our experiments provide comparative insights as to how a spinal microcircuit centered on a particular cell class but mediating different forms of motor activity develops during the maturation of motor control.

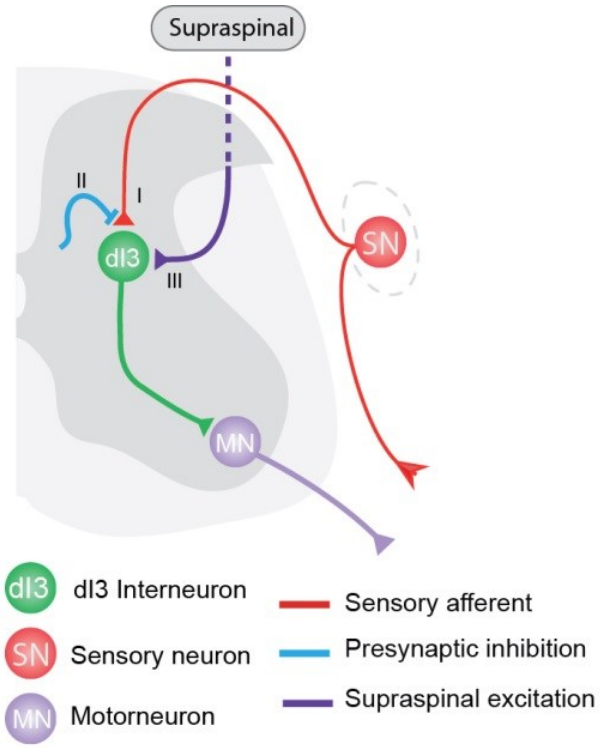

Figure 2. Diagram of the connectivity of dI3 INs and possible pathways involved in motor maturation.
i) sensory afferents contacting dI3 INs (ii) putative presynaptic inhibition of sensory afferents contacting dI3 INs (iii) putative supraspinal excitation of dI3 INs.

CHAPTER 2
Connectivity of dI3 Interneurons during the development of mice spinal cord

Carl Farah, Tuan V. Bui

2.1 Introduction

In early development, neural circuits within the spinal cord can autonomously produce coordinated movements. Locomotion, which can be observed as early as embryonic and postnatal stages (Altman and Sudarshan 1975; Branchereau et al. 2000), is perhaps the best example of a behaviour that can be expressed solely by the spinal cord. However, the maturation of motor control, including movements that can be autonomously generated by spinal circuits, relies on the maturation of key inputs to the spinal circuitry (Dominici et al. 2010; Lacquaniti et al. 2012).The integration of sensory information relayed by peripheral afferent in the spinal cord and its gating by presynaptic inhibition has been implicated in the production of smooth and coordinated movements (Akay et al. 2014; Fink et al. 2014). The development of descending pathways also contribute to the maturation of motor control and the developmental implications of the corticospinal tract (CST) in motor control have received much attention (Porter and Lemon 1993; Lemon and Griffiths 2005; Martin 2005).

The development of key inputs to the spinal cord seems to progress with different timelines. Dorsal root projections conveying sensory information to the spinal cord reach the ventral horn by embryonic day (E) 15.5 (Ozaki and Snider 1997). In the cat, these sensory connections are capable of eliciting monosynaptic reflexes towards the end of fetal development (Naka 1964). On the other hand, presynaptic inhibition of primary afferents contacting MNs appear by E16.5 but become functional only after the first week of development (Betley et al. 2009). Similarly, CST development continues postnatally and labelling studies have revealed

that the CST first reaches the cervical and lumbar segments by postnatal day (P) 0 and 6, respectively, with collaterals extending further by P8 (Bareyre et al. 2005).

While sensory inputs are the first to extend into the spinal cord, sensory innervation is continually fine-tuned during postnatal development. During the first three postnatal weeks, there is continual sprouting and elimination of afferent connections as such that laminar termination of primary afferents to ventral, intermediate and dorsal laminae of the spinal cord decrease considerably after birth in mice (Fitzgerald et al. 1994; Granmo et al. 2008). Another such example of sensory refinement is evidenced in the circuitry responsible for the nociceptive withdrawal reflex (NWR), which in early postnatal stages undergoes refinement of NWR modules which integrate incoming sensory information (Ladle et al. 2007). Therefore, while sensory inputs are continually refined during postnatal stages, motor control undergoes its own maturation, suggesting that both processes are linked.

In addition to changes in sensory inflow through refinement of sensory processes innervating the spinal cord, changes in gating of sensory transmission such as by the aforementioned presynaptic inhibition are also present. Presynaptic inhibition, which acts to limit transmission by sensory afferents through direction inhibition of sensory terminals within the spinal cord, has been demonstrated to be essential in executing skilled movements such as reaching (Fink et al. 2014). Genetic labelling of Ptf1a, which specifies a population of GABAergic presynaptic inhibitory (GABApre) interneurons, has demonstrated that the first two weeks of development are necessary for the maturation of the presynaptic inhibition circuitry (Betley et al. 2009). Furthermore, CST inputs have been shown to contact GABApre neurons

mediating presynaptic inhibition (Russ et al. 2013) and this connectivity may also be involved in furthering the maturation of motor control.

Beyond contacting GABApre interneurons, the CST terminates on many spinal neurons, and proper development of the CST is known to contribute to the maturation of fine motor control such as reaching and the dexterous manipulation of objects (Porter and Lemon 1993; Lemon 2008). Direct connections between cortical neurons and spinal motoneurons, observed in primates, from the CST have been suggested to contribute to finer hand movements (Riddle et al. 2009). The CST is very much involved in the refinement of sensorimotor integration as its development is implicated in modulating spinal reflexes (Bretzner and Drew 2005) as well as postsynaptic inhibition of spinal interneurons mediating local (Canedo 1997; Bedell et al. 2014) and cortical sensorimotor integration via ascending spinal pathways (Hantman and Jessell 2010; Moreno-López et al. 2016). Additionally, CST inputs have been implicated in mediating long-term plasticity of spinal circuits (Wolpaw 1997) through spinal interneurons. However, the extent of CST innervation in the spinal cord, especially towards spinal interneurons mediating motor behaviours is not well established.

So far, the development of incoming spinal inputs and how it relates to the maturation of motor control has primarily been studied in motoneurons (MNs). For instance, MNs have been observed to undergo substantial sensory pruning during development (Gibson and Clowry 1999; Gibson et al. 2000). As the final common pathway, MNs ultimately propagate the centrally-derived patterns of activity necessary for muscle activation, however, each MN receives synaptic inputs from an array of upstream premotor spinal interneurons (Brownstone and Bui 2010). In

addition, these spinal interneurons receive their share of sensory and descending inputs. Therefore, maturation of motor control likely involves the development of inputs to the premotor spinal interneurons and invaluable insights into the maturation of motor control can be gained from the study of this development.

To our knowledge, the development of connectivity to specific spinal interneurons has only been studied for a small number of interneuron population such as Renshaw cells (Mentis et al. 2006). However, it is difficult to interpret how the development of the inputs to these specific interneuron populations relates to the maturation of motor control without a clear understanding of the contributions of these spinal neurons to motor control. Only recently has the study of spinal interneurons benefited from the identification of specific sets of molecular markers to differentiate distinct populations of spinal neurons (Alaynick et al. 2011). The use of genetic techniques to study different sets of spinal interneurons has led to many insights into their respective functional roles. Presently, the study of genetically identified spinal interneurons has largely focused on populations of neurons pertaining to locomotion (Lu et al. 2015). But important advances have been now been made in identifying populations of neuron contributing to diverse motor tasks such as those involved in grasping, fine motor control, limb position during locomotion and smooth movement production (Bui et al. 2013; Fink et al. 2014; Bourane et al. 2015; Hilde et al. 2016).

Recent work has established that one of these spinal populations, dI3 interneurons (dI3 INs), forms a sensorimotor circuit integrating cutaneous low-threshold mechanoreceptors and proprioceptors for grasping (Bui et al. 2013). Genetic silencing of dI3 INs led to a marked

decrease of the palmar grasp reflex (PGR) in neonates accompanied by a significant loss of cutaneous grasping in adults (Bui et al. 2013). This implies that maturation of the control of the PGR through dI3 INs, may lead to the refinement of grasping in adults. In light of their developmental implications in grasping, dI3 INs are ideally positioned as candidates for the study of refinement of hand control.

In this study, we aimed to quantify the changes in sensory transmission through sensory afferentiation, inhibitory gating of sensory information as well as corticospinal excitation to dI3 interneurons. To relate changes in connectivity of dI3 INs to maturation of motor control, we further determined the developmental time point at which the PGR disappears. We show that sensory afferentiation onto dI3 INs is consistent during maturation and that presynaptic inhibition of primary afferents increases during development. Moreover, the developmental time course of the PGR does not appear to perfectly match that of the presynaptic inhibition of afferents contacting dI3 INs, suggesting that the development of presynaptic inhibition to sensory afferents contacting dI3 INs is not the main impetus for the disappearance of the PGR. In addition, we show that CST terminals contact dI3 INs and that corticospinal innervation decreases with maturation. These changes in connectivity of dI3 INs were compared for cervical and lumbar dI3 INs as a means to compare the development of cervical and lumbar motor function. Our results show that there is some refinement of connectivity of dI3 INs during postnatal development.

2.2 Methods

2.2.1 Animals

Expression of YFP driven by the promoter for the homeodomain transcription factor Isl1 was obtained in double transgenic offspring of Isl1-Cre and Rosa26-lox-stop-lox-YFP mice which are henceforth known as Isl1: YFP. All animal procedures were approved by the University Committee on Laboratory Animals of the University of Ottawa and conform to the guidelines put forth by the Canadian Council for Animal Care. Animals used in experiments were of ages ranging from postnatal (P) 3 to 60 which were considered adults. Cervical segments innervating the forepaws studied were distributed between C5-C8. Lumbar segments innervating the hind paws are distributed between L1-L6. Both male and female mice were used in this study.

2.2.2 Genotyping

The following primers were employed in genotyping Isl1:YFP mice. Wildtype Reverse CRE (5′-CAAATCCAAAAGAGCCCTGTC-3′), Isl1-Cre Forward (5′-GCCACTATTTGCCACCTAGC-3′), and Mutant Reverse (5′-AGGCAAATTTTGGTGTAC-3′). Rosa26 oligo 1 (5′-AAAGTCGTCTGAGTTGTTAT-3′), Rosa26 oligo 2 (5′-GCGAAGATTTGTCCTCAACC-3′), Rosa26 oligo 3 (5′-GGAGCGGGAGAAATGGATATG-3′).

2.2.3 Palmar Grasp Reflex Testing

To test for the presence of the palmar grasp reflex (P7-21), a small tubular rod was stroked on the palmar surface of the paw while any flexion of the fingers was observed. Successful elicitation of the PGR was defined as the reflexive closing of the fingers around the rod. Each mouse was subjected to one stimulation trial on each day of testing consisting of a single presentation of the stimulus to the forepaw.

2.2.4 Immunohistochemistry

Mice were transcardially perfused with ice-cold 4% paraformaldehyde and their spinal cords were removed and postfixed overnight. Tissue was cryoprotected in 30% sucrose and transverse sectioned on a cryostat at (50µm) and collected as free-floating sections. Sections were washed three times with PBS, blocked for 1h in PBS with 0.25% triton-X100 (PBST) and 5% serum, and then incubated overnight at 4°C with primary antibodies in PBST plus 5% serum. The following day, sections were washed three times with PBS and incubated with Alexa Fluor-conjugated secondary antibodies for three hours at room temperature and washed three final times in PBS. Antibodies used for fluorescent immunohistochemistry were as follows: rabbit anti-GFP (1:1000; Abcam), sheep anti-GFP (1:1000; Bio-Rad), guinea-pig anti-vGLUT1 (1:2500; EMD Millipore), guinea-pig anti-vGLUT2 (1:2500; EMD Millipore), mouse anti-GAD65 (3µg/mL; Developmental Studies Hybridoma Bank), and mouse anti-PKCγ (1:100; Santa Cruz). Sections were mounted onto superfrost slides with Immu-Mount and coverslipped.

2.2.5 Synaptic quantification

Terminals were quantified on the entire soma on confocal images in the z-axis collected by a Nikon A1MP confocal microscope equipped with four single laser lines. Nikon NIS-Elements Viewer Software and ImageJ were employed for image analysis. Axo-somatic (sensory afferent and corticospinal inputs) and axo-axonic (presynaptic inhibitory inputs) connections were manually identified using orthogonal views to visually confirm apposition boutons.

2.2.6 Statistical Analysis

Unless otherwise noted, data are reported as mean ± SD. Groupwise comparisons were performed using a one-way ANOVA with a threshold of significance set at 0.05. Pairwise comparisons were performed using Student's unpaired t-test with unequal variance where applicable and a threshold for significance adjusted using a post hoc bonferroni correction.

2.3 Results

2.3.1 Loss of PGR during development

We first set out to determine at what developmental time point the PGR disappears. The PGR first appears around P3 and can be robustly elicited from P7 onwards (Fox 1965). The PGR was elicited in Isl1:YFP transgenic mice from P7 to P21 using a previously validated method (Bui et al. 2013).The PGR was still present at P7 and could be consistently elicited until P18, by P19-20, a sharp decrease (50%) in incidence was observed (Figure 3). Finally, by P21, the PGR could not be elicited.

2.3.2 dI3 INs receive corticospinal input

Our results above confirm prior observations that the PGR disappears during postnatal development in mice. With the knowledge that dI3 INs are essential for the PGR, we sought to characterize which development changes could contribute to the disappearance of the PGR during maturation. In view of the contribution that the CST makes to the execution of voluntary movements (Martin 2005; Lemon 2008) and in particular grasping (Alstermark and Pettersson 2014), we first asked whether CST neurons synapse directly onto dI3 INs of the cervical spinal cord. We identified dI3 INs by yellow fluorescent protein (YFP) expression in Isl1:YFP transgenic mice (Bui et al. 2013). The majority of CST terminals are known to express both vesicular glutamate transporter 1 (vGLUT1) (Du Beau et al. 2012) as well as Protein Kinase C γ (PKCγ) (Hantman and Jessell 2010; Russ et al. 2013). We observed $PKC\gamma^{+}$/$vGLUT1^{+}$ boutons synapsing onto dI3 INs (Figure 4Ci-Cvii). By p7, we observed no PKCγ/vGLUT1 co-labelling although PKCγ INs in lamina II were detectable thus suggesting that CST terminals do not express PKCγ at this developmental time point. By p14, we observed that 100% of YFP^{+} dI3 INs

(n= 17 cells, two animals) receive input from PKCγ^{+}/vGLUT1^{+} CST terminals (3.6 ± 1.3 boutons /dI3 IN soma and proximal dendrites, n= 17) (Figure 4D-E). By p28, we observed that 100% of YFP^{+} dI3 INs (n = 8 cells, two animals) receive input from PKCγ^{+}/vGLUT1^{+} CST terminals (3.1 ± 2.4 boutons /dI3 IN soma and proximal dendrites, n= 8). In adults, we observed that 78% of YFP^{+}dI3 INs (n = 11 out of 14 cells, two animals) receive input from PKCγ^{+}/vGLUT1^{+} CST terminals (1.9 ± 0.9 boutons /dI3 IN soma and proximal dendrites, n=11). When plotted over age, the development of CST input onto dI3 INs. It is worth mentioning that we observed PKCγ^{+} boutons devoid of vGLUT1 staining on dI3 INs (Figure S1). These boutons likely stem from PKCγ^{+} spinal INs found in the dorsal horn involved in gating innocuous input (Neumann et al. 2008).

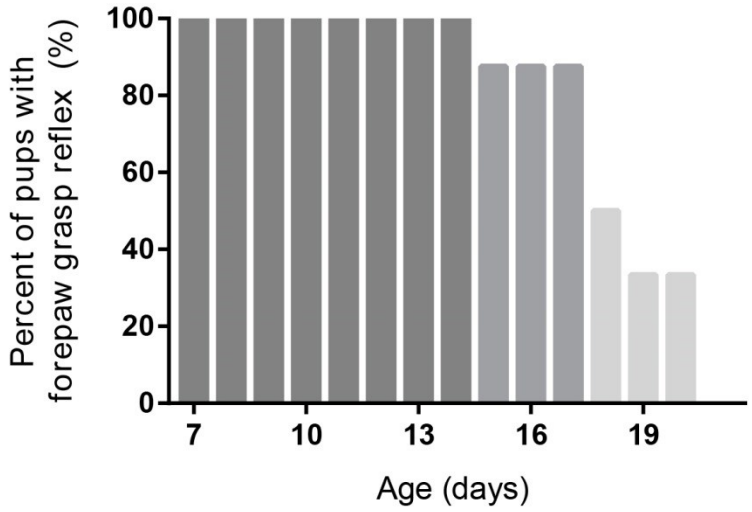

Figure 3. Incidence of the palmar grasp reflex during development.
Mice (n=6) were subjected to one trial of testing each day.

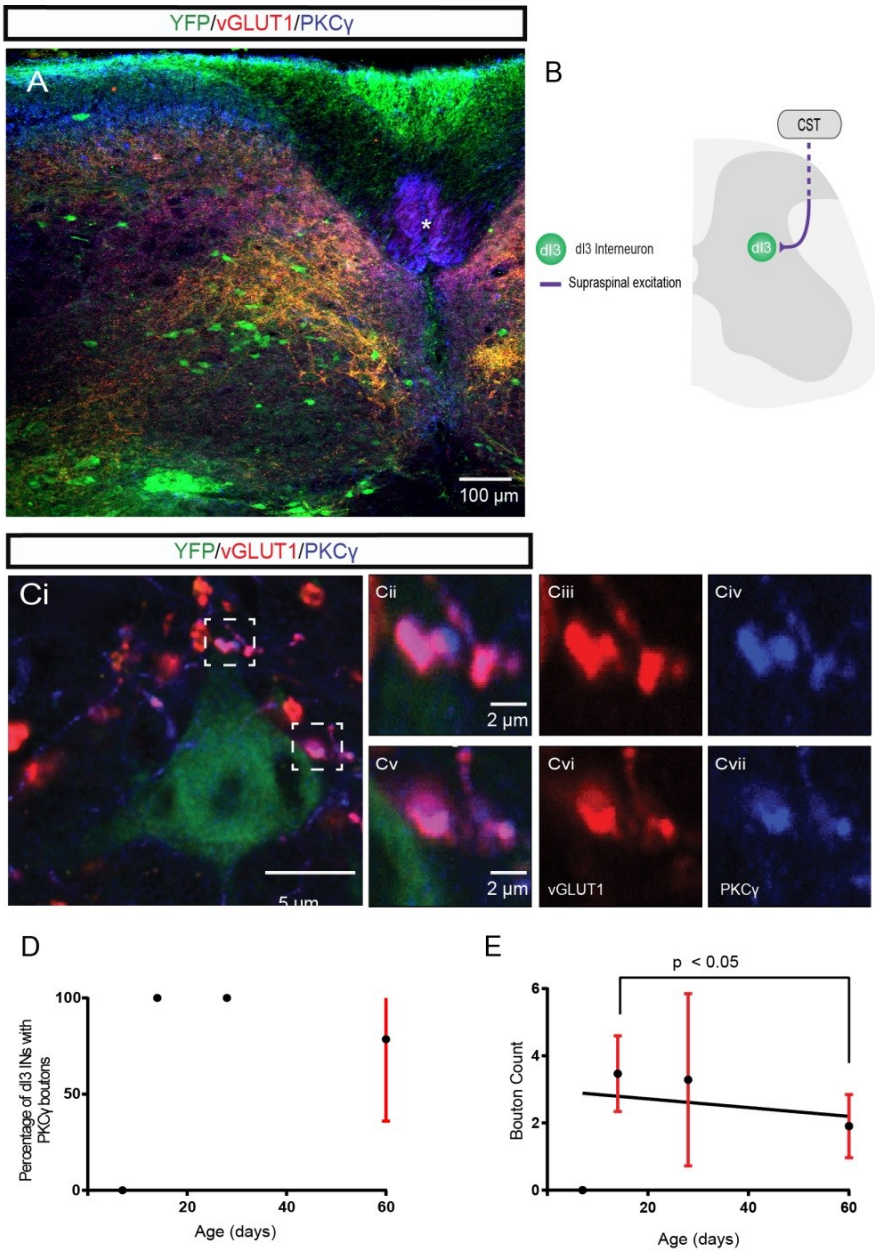

Figure 4. Development of CST inputs to dI3 INs.
(A) Distribution of cervical YFP$^+$ dI3 INs and PKCγ$^+$/vGLUT1$^+$ terminals in an adult transverse spinal cord section. asterisk shows staining where the dorsal corticospinal tract is expected to be located, supporting the accuracy of our PKCγ staining.
(B) Diagram representing supraspinal connectivity to dI3 INs.
(C) PKCγ$^+$/vGLUT1$^+$ boutons contact a cervical dI3 IN from a P14 spinal cord. Boutons in dashed boxes are magnified in (Cii)-(Cvii).
(D) Percentage of cervical dI3 INs contacted by PKCγ$^+$/vGLUT1$^+$ terminals during development. Data are plotted as mean ± SD.
(E) Number of PKCγ$^+$/vGLUT1$^+$ boutons contacting cervical dI3 INs during development. Data are plotted as mean ± SD and fitted to a linear regression (n = 2 mouse for each group, $r^2 = 0.02$).

2.3.3 Development of Primary afferent innervation

During maturation, mechanisms such as presynaptic inhibition of primary afferents among others may contribute to reduced sensory excitation of dI3 INs and lead to the disappearance of the PGR. However, it remains unknown which of these development changes, if any, are responsible for the loss of this particular reflex. We asked how sensory afferentiation onto dI3 INs might change during development. Vesicular glutamate transporter 1 (vGLUT1) expression has been known to characterize sensory terminals. Given the small CST ($PKC\gamma^+/vGLUT1^+$) contribution onto dI3 INs, vGLUT1 was employed to identify sensory terminals onto dI3 INs (Figure 5C). The number of $vGLUT1^+$ boutons contacting dI3 INs did not significantly change during development (Figure 5D). During maturation, dI3 INs from cervical segments were contacted by 3 to 20 $vGLUT1^+$ boutons (10.1 ± 8.4 boutons/dI3 IN soma, n = 78 cells) while dI3 IN from lumbar segments were contacted by 5 to 28 $vGLUT1^+$ boutons (10.7 ± 7.9 boutons/dI3 IN soma, n = 76 cells). The only significant difference in sensory afferentiation between spinal segments was observed at age P15 where cervical and lumbar segments were contacted by 8.83 ± 0.9 (n =12 cells) and 11.9 ± 0.9 (n=11 cells) $vGLUT1^+$ boutons/dI3 IN soma, respectively.

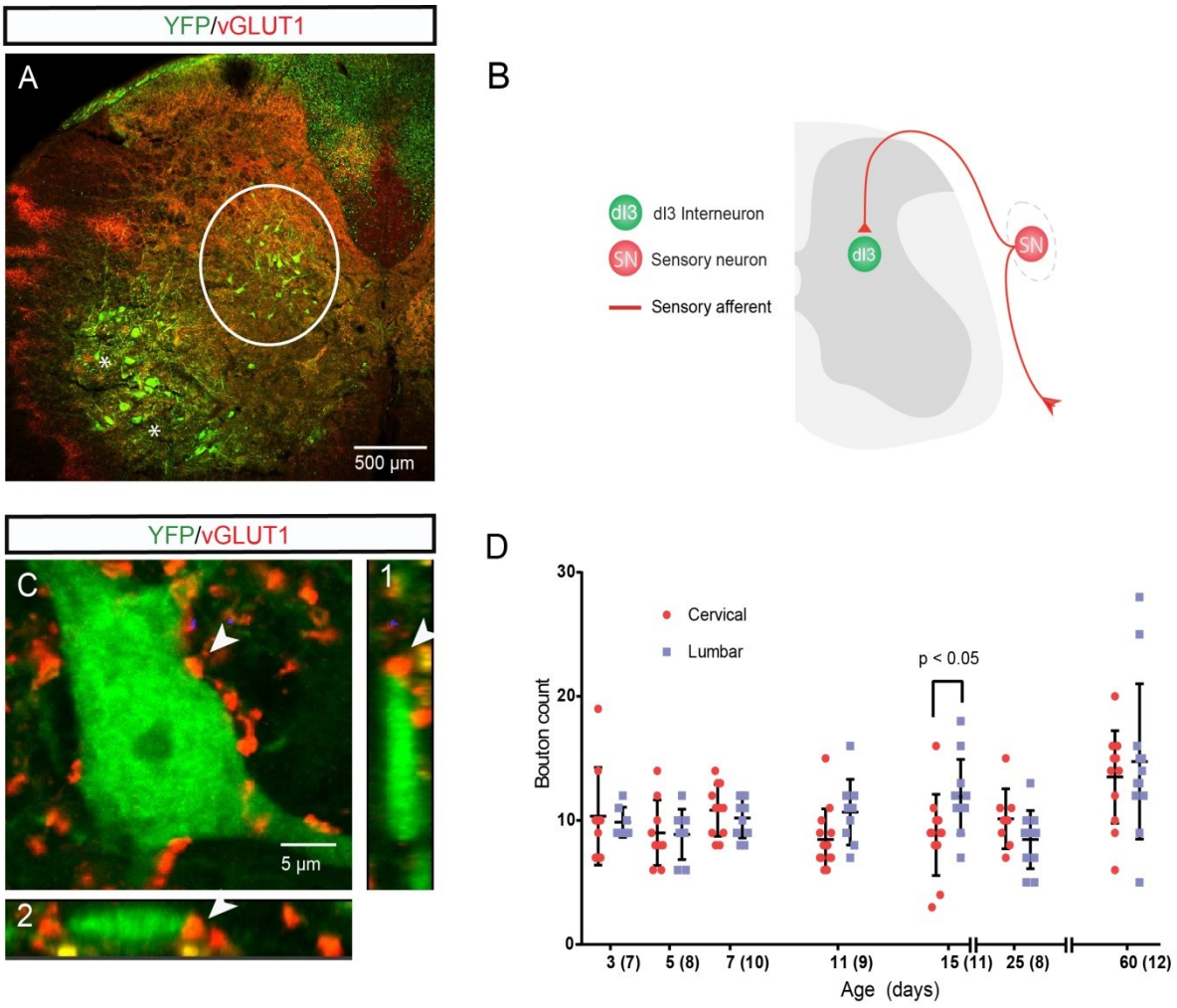

Figure 5. Development of primary afferents contacting dI3 INs.
(A) Distribution of cervical YFP⁺ dI3 INs and vGLUT1⁺ terminals in an adult transverse spinal cord section. dI3 INs are shown within the circle and an asterisk denotes the location of motoneurons.
(B) Diagram representing the connectivity of sensory afferents to dI3 INs.
(C) vGLUT1⁺ boutons (arrowhead) contact a cervical YFP⁺ dI3 IN from an adult spinal cord. Orthogonal sections confirm apposition of labelled bouton 1-2.
(D) Number of vGLUT1⁺ boutons contacting cervical and lumbar dI3 INs is plotted over age with the number of neurons visualized at each age group in parantheses. Data are plotted as mean ± SD (n = 2 mouse for each group)

2.3.4 Primary afferents contacting dI3 INs receive presynaptic inhibitory inputs

Considering that sensory afferentation seemed to remain constant during development, we turned our attention to presynaptic inhibition as a mechanism for attenuating sensory transmission to dI3 INs. In view of the importance presynaptic inhibition plays in the execution of smooth movement and grasping (Fink et al. 2014), we asked whether inhibitory axo-axonic connections were present on primary afferents contacting dI3 INs of both the cervical and lumbar spinal cord. We used GAD65 as a molecular marker for presynaptic inhibition with the knowledge that GABApre terminals express both GAD65 and 67 and are opposed to sensory terminals labelled by vGluT1 while GABApost terminals solely express GAD67 and are observed on the soma (Betley et al. 2009). To quantify GABApre terminals, we calculated the percentage of $GAD65^+$ contacts on $vGLUT1^+$ boutons synapsing onto dI3 INs (Figure 6Ci-Cvii).

Genetic labelling of Ptf1a, which specifies a population of GABApre neurons, determined that at birth, axo-axonic contacts are present on sensory terminals but are devoid of associated synaptic markers (Betley et al. 2009). Synaptic markers such as GAD65/67 for presynaptic inhibitory terminals were shown to appear after the first week of development (Betley et al. 2009).We asked how the prevalence of inhibitory axo-axonic connections onto primary afferents contacting dI3 INs might change during maturation. From early postnatal development to adulthood, $GAD65^+$ coverage of primary afferents (labelled by vGLUT1) contacting dI3 INs ranged from 43.8 ± 14.5 to 88.2 ± 3.7%. More than half of the age groups for both cervical and lumbar segments showed significant differences in the incidence of presynaptic inhibition during development (Table S1). When plotted over age, the development of presynaptic inhibitory terminals onto primary afferents contacting dI3 INs followed a one-phase

exponential relationship yielding an r-squared of 0.51 and 0.56 for cervical and lumbar segments, respectively (Figure 6D). Previous work suggests that at P0, the spinal cord is devoid of molecular markers associated to presynaptic inhibition (Betley et al. 2009). Therefore, incorporation this observation increased the r-squared to 0.77 and 0.81 for cervical and lumbar segments, respectively (Figure 6E). Development of presynaptic inhibitory terminals was similar for both cervical and lumbar segments throughout development.

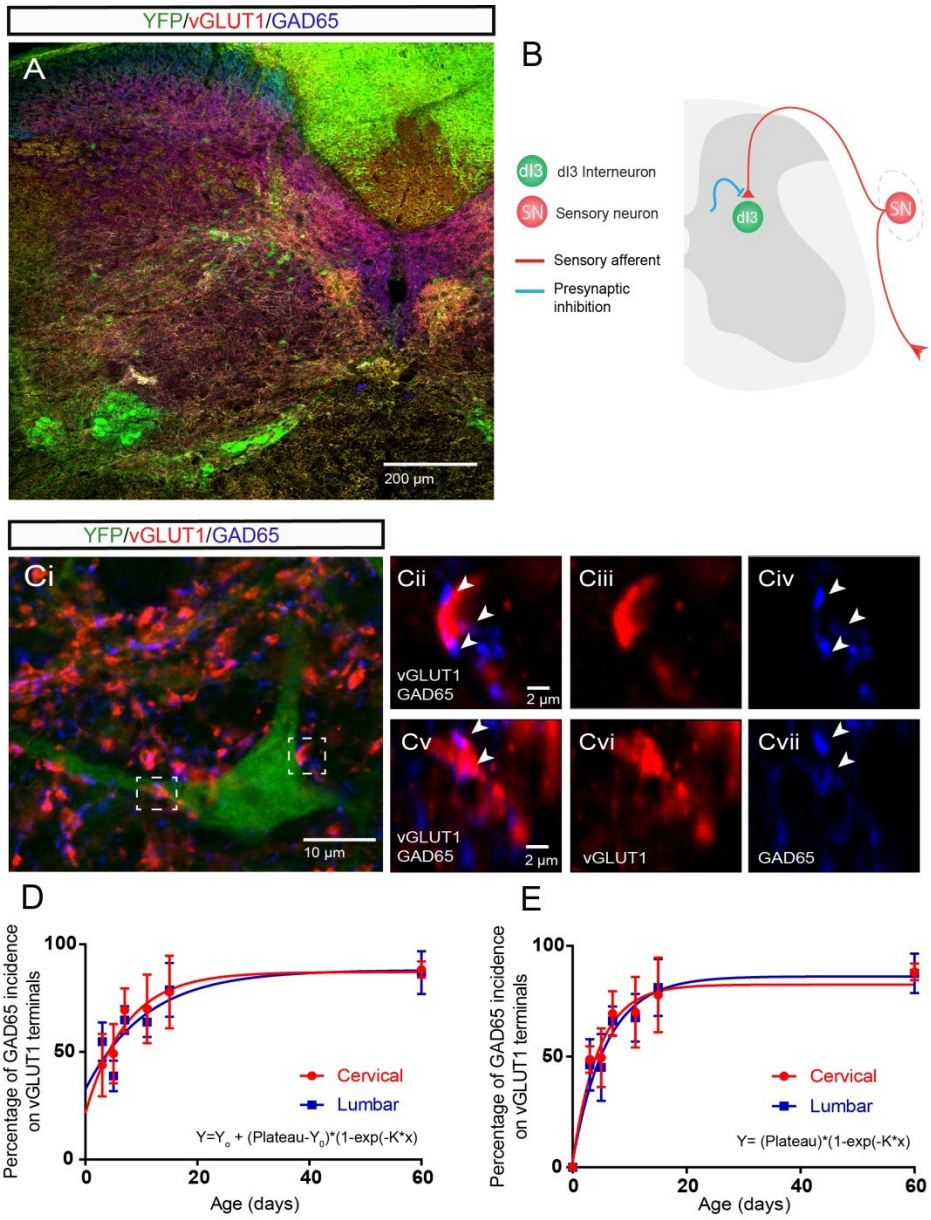

Figure 6. Development of presynaptic inhibition of primary afferents contacting dI3 INs.
(A) Distribution of cervical YFP$^+$ dI3 INs and vGLUT1$^+$/GAD65$^+$ terminals in an adult transverse spinal cord section.
(B) Diagram representing presynaptic inhibition of sensory afferents contacting dI3 INs.
(C) GAD65$^+$ terminals contact vGLUT1$^+$ boutons on a cervical dI3 IN from an adult spinal cord. Boutons in dashed boxes are magnified in (Cii)-(Cvii) where arrowheads depict the GAD65$^+$ terminals.
(D) Percentage of primary afferents on dI3 INs contacted by presynaptic inhibition during development represented as mean ± SD and fitted to an exponential (one-phase association) (n = 2 mouse for both groups), $r^2 = 0.51$ and 0.58 for cervical and lumbar, respectively.
(E) Data from (D) is re-plotted with observations from Betley et al. 2009, $r^2 = 0.77$ and 0.78 for cervical and lumbar, respectively.

2.4 Discussion

We have determined the developmental time course of different inputs onto a particular cell class mediating grip. Our analysis of key inputs to dI3 INs reveals that the amount of sensory innervation remains constant and that the amount of presynaptic inhibitory terminals increases during postnatal development. However, the development of these inputs does not completely match the timeframe in which the PGR disappears. This suggests that a quantification of the development of sensory and presynaptic inhibitory terminals onto dI3 INs is not sufficient to provide a full understanding of the mechanism underlying the disappearance of the PGR. In addition, we observed CST inputs on dI3 INs and show that these inputs decrease during maturation.

2.4.1 Methodological Considerations

Due to the observed small size of primary afferent varicosities in younger animals, it is possible to have underestimated the actual number of terminals present. In addition, it is interesting to note that we observed a lack of PKCγ staining at early developmental time points when the CST is known to innervate the cervical segment (Donatelle 1977) suggesting that PKCγ is not yet expressed by CST terminals. We also report that at P15, the lumbar segment received significantly greater sensory innervation then its cervical counterpart. It may be possible that this particular finding is a result of outlier data points arising due to under sampling. Finally, it is important to recognize that due to methodological constraints, this study solely focused on including synapses that contacted the cell body. Therefore, the connective patterns observed on cell bodies may differ from those observed on dI3 INs dendritic arbors.

2.4.2 Ontogeny of primitive reflexes

Primitive reflexes have been known to disappear during development of the central nervous system and reappear when supraspinal mediated inhibition is released such as seen in patients of cortical lesions (Futagi et al. 2012). Little is known about the function of primitive reflexes other than being a set of developmentally conserved behaviours that promote survival. Few studies have investigated whether the circuity employed by primitive reflexes is conserved during development and/or the roles served by these circuits within a mature nervous system. Previous reflex studies have established that the PGR can be robustly elicited from P7 onwards (Fox 1965), however, the disappearance of this particular reflex has not been well documented. Our findings suggest that the PGR begins to disappear around P18 and is completely absent by P21.These data suggest that important developmental changes may occur during this critical time period which ultimately leads to the disappearance of the PGR.

Genetically identified interneurons provide an excellent opportunity to study how motor control matures (Bui et al. 2015). Several interneurons such as the nuclear orphan receptor (RORα) (Bourane et al. 2015), Satb2 (Hilde et al. 2016) and dI4 (Fink et al. 2014) IN populations have been involved in sensorimotor control. Un-identified spinal INs (Takei and Seki 2013) that may or may not include the aforementioned interneurons, have been found to be implicated in the production of precision grip in monkeys. However, dI3 INs distinguish themselves as being the only population to have experimentally identified distinct but related roles in postnatal and mature development (Bui et al. 2013). It is possible that in light of the role Satb2 INs play in nociception (Hilde et al. 2016), that the nociceptive withdrawal reflex at postnatal stages could be affected, however, this remains untested.

Sensory refinement has been implicated in the development of certain reflexes, namely the cutaneous flexion withdrawal reflex (CFWR). Indeed, the CFWR has been shown to be mediated by low-threshold fibers in neonates but by high-threshold fibers in the adult (Sherrington 1910; Fitzgerald et al. 1988). This development prevents flexor movements to be elicited by mere innocuous touch. Further, changes in sensory processing incurred during development such as reduced receptive field size or appearance of presynaptic inhibition support the idea that as the nervous system develops it becomes less reactive in nature (Altman and Bayer 2001). Developmental studies of sensory afferentiation have largely focused on MNs and have determined that maturation leads to a substantial amount of sensory pruning (Gibson and Clowry 1999). This pruning further supports the idea that maturation is accompanied by a state of reduced sensory excitability. We hypothesized that maturation of grasping, which leads to the disappearance of the PGR and the appearance of volitional grasping, may have led to reduced sensory afferentation and therefore reduced excitation of dI3 INs by sensory inputs. However, our findings suggest that sensory afferents remain constant during development, for both cervical and lumbar segments. Previous electrophysiological experiments determined that sensory input to dI3 INs decreased in latency between P7 and P15 (Bui et al. 2015). With the knowledge that dI3 INs receive constant sensory afferentiation during development, it is now possible to confirm that these stimulation latencies are not due to a shift from polysynaptic to monosynaptic sensory inputs to dI3 Ins but were in fact due a lack of myelination (Webster 1971).

Few studies have compared the amount of sensory afferents terminating on cervical and lumbar sub-populations of a particular class of spinal neurons. One could expect differences

based upon the overall differences in motor tasks under the control of cervical and lumbar spinal networks. Indeed, cervical segments are more strongly associated with higher skilled dextrous movements whereas the lumbar segments mediate more coarse and subconscious movements such as locomotion. For example, cervical dI3 INs integrate sensory information for fine grip whereas lumbar dI3 INs integrate sensory information for proper paw placements of the limbs during locomotion. It is thus tempting to hypothesize that dI3 INs in the cervical segments responsible for fine grip would receive greater sensory innervation then those found in the lumbar segments, which may be more predominantly dedicated to coarse grip or locomotor activity. However, our study suggests that the development of sensory afferentation is similar for both cervical and lumbar segments. While both sub populations of dI3 INs integrate sensory information for different motor tasks, our data suggest they receive similar number of sensory afferents.

If sensory pruning does not seem to account for reduced sensory excitation of dI3 INs, perhaps mechanisms gating sensory transmission such as presynaptic inhibition could account for reduced sensory transmission to dI3 INs. The importance of presynaptic inhibition in the production of smooth movement, particularly in those involving reaching and grasping motions has been eloquently demonstrated by genetic ablation of these interneurons (Fink et al. 2014). Further, this filtering mechanism is extensively associated with spinal sensorimotor circuits as evidenced by the finding that approximately 90% of primary afferents contacting MNs are themselves subject to presynaptic inhibition (Betley et al. 2009). Although presynaptic inhibition centered on a particular spinal interneuron class has not yet been studied, previous developmental studies have determined that the associative molecular markers for presynaptic

inhibition such as GAD65/67 are absent at birth and are expressed on sensory terminals contacting motoneurons after the first week of development (Betley et al. 2009). Our data are in accordance with these observations and suggest that presynaptic inhibition onto primary afferents contacting dI3 INs increases in the days following birth and develops along an exponential relationship with age. Indeed, incorporating previous observations made at birth (Betley et al. 2009) with our data on the developmental of presynaptic inhibition to dI3 INs improves the fit of the relationship of presynaptic inhibition to dI3 INs with age (Figure 5E). Although the immunohistochemical developmental time course of presynaptic inhibition does not completely match the disappearance of the PGR, we cannot discount that the magnitude of presynaptic inhibition, by way of varying synaptic strength of GABApre terminals, may be a perfect match after all.

2.4.3 Descending control of primitive reflexes

The integration of higher brain centers is essential in producing well-coordinated movements (Porter and Lemon 1993; Lemon 2008). Early development of descending tracts is accompanied by an overgrowth of CST axons in the spinal cord and as development progresses, there is substantial elimination of these terminals (Bates and Killackey 1984; Curfs et al. 1994). Indeed, the CST reaches the cervical segment by birth and pruning begins as early as the second postnatal week (Curfs et al. 1994). Labelling studies in the rat have revealed that the CST innervates the cervical segments most intensely at P10 and that pruning can be observed from the second week onwards (Curfs et al. 1994). In the cat, CST maturation is accompanied by an increase in branch number but a decrease in length, an increase in varicosity density and a decrease in termination field (Qun and Martin 2011). We show that dI3 INs, by way of the CST, likely integrate descending commands for grip. Even though our technique for identifying CST

terminals using PKCγ staining only allows us to resolve these terminals after the first week as PKCγ staining is absent before this age, our data regarding CST input to dI3 INs seems to be in accordance with the time course of CST maturation in the spinal cord.

Several spinal interneurons involved in sensorimotor integration such as the ROR alpha (RORα) nuclear orphan receptor interneurons (Bourane et al. 2015), dorsal spinocerebellar tract (dSC) interneurons (Hantman and Jessell 2010) and presynaptic inhibition interneurons (Rudomin and Schmidt 1999) populations have been known to receive CST input. These findings lend further credence to the idea that the CST is implicated in the maturation of motor control through the development of the production of skilled and well-coordinated grasping movements. Recent studies have determined that Ptf1a-labelled dI4 INs account for roughly 90% of GABApre terminals onto primary afferents (Betley et al. 2009). In addition, these INs have also been found to receive input from the CST (Russ et al. 2013), however, how CST inputs to dI4 INs develop remains to be determined. The development of this particular connection may be very important in the maturation of motor control. Further, the functional subdivision of the entire dI4 IN population have not been characterized yet and the possibility exists that there are subpopulations of dI4 INs dedicated to gating sensory transmission to dI3 INs, the development of which would be of particular relevance to the maturation of grasping.

Whether the development of GABApre terminals onto sensory afferents relies on dI4 INs receiving CST input remains to be determined. dI4 INs axons can be observed in the dorsal horn as early as embryonic day 12.5, however, molecular markers of synaptic release appear around postnatal day 3 (Betley et al. 2009). Recent studies have demonstrated that BDNF and glutamate

release from sensory afferents regulates expression of both GAD65 and 67 in GABApre terminals, respectively (Mende et al. 2016). In spite of the CST reaching the cervical and lumbar segments at different time points, our data indicates that presynaptic inhibition develops similarly in cervical and lumbar segments, suggesting that the number of presynaptic inhibition terminals is not fully dependant on the CST. The findings from Mende et al. (2016) and the discrepancies in developmental time courses of CST and presynaptic inhibition suggests that that development of GABApre neurons depends to larger extent on the release of BDNF by sensory afferents in the spinal cord then on development of the CST. However, it is possible that CST maturation alters the synaptic weight or strength of synaptic transmission of GABApre terminals.

2.4.4 Future directions

We have shown that sensory afferentiation, by virtue of number of sensory terminals, reaching a critical interneuron involved in grasping remains constant during development. In addition, we have demonstrated that presynaptic inhibition of these sensory afferents increase during age but cannot solely explain the disappearance of the PGR. Finally, we showed that these interneurons receive corticospinal input and that this input decreases during maturation. In brief, studying the development of the CST onto dI4 INs may lead to insights about their involvement in the disappearance of the PGR through sensory gating of dI3 IN sensory inputs. Given that prior studies of both dI3 and dI4 INs make use of conditional expression of CRE, future studies of the development of descending control of presynaptic inhibition specific to sensory inputs to dI3 INs may rely upon intersectional genetic strategies involving crossing one of the CRE driver line with a newly generated FLP-FRT or I-SceI construct to conditionally express a different reporter gene for the other interneuron population of interest. Alternatively, electrophysiological preparations where adjacent dorsal roots are stimulated (Mende et al. 2016)

could be used to further probe the role presynaptic inhibition plays in the sensorimotor circuit involving dI3 INs. Further understanding of the multitude of inputs dI3 INs receive and how these inputs change during maturation will add upon our results to deepen an understanding of the maturation of a spinal circuit dedicated to a particular motor function.

2.5 Acknowledgements

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CHAPTER 3
Discussion

This thesis aimed to provide insights into the maturation of motor control at the level of the spinal cord through development of connectivity of spinal circuits. To reach this greater aim, I chose to focus on the developmental time course of the primitive palmar grasp reflex and attempted to use the development of key inputs to dI3 INs, the population of spinal neurons believed to underlie this reflex, to shed light on the loss of this reflex. Using immunohistochemical methods, I examined the development of primary afferents onto dI3 INs in both cervical and lumbar segments. Next, I identified and studied the development of presynaptic inhibition onto these sensory afferents. Finally, I was able to investigate the possibility of corticospinal input to cervical dI3 INs.

I was able to determine that the palmar grasp reflex is absent by the third postnatal week. In addition, I found that sensory afferentiation and more precisely the number of sensory terminals onto dI3 INs in both cervical and lumbar segments remained constant during development. Further, I established that sensory afferents contacting dI3 INs received presynaptic inhibition and that this inhibition increased during maturation. Finally, I found that dI3 INs receive decreasing corticospinal input during development (Figure 7). Although these inputs do not appear to solely account for the disappearance of the PGR based upon the time course of the development of their innervation of dI3 INs, they are able to shed light on the maturation of the circuitry responsible for this particular reflex.

Motor control is continuously refined during development, from the increased dexterity in hand control of a child to the development of locomotion. Suggested as the most complex

structure in the universe (Hawryluk et al. 2012), the CNS holds many of the structures and populations necessary for proper motor control. Indeed, while there are many different approaches to study the neural circuitry underlying motor control, recent advances in the identification of molecular markers of neurons and the use of genetically identified neurons has led to many new insights about locomotion (Rybak et al. 2015), grasping (Bui et al. 2013) and sensorimotor integration (Fink et al. 2014). The ability to relate specific behaviours to populations of identified of neurons has proven essential in attributing a particular role to such populations.

Among these genetically identified spinal interneurons, dI3 INs have been shown to mediate the PGR in early development and grasping in adults (Bui et al. 2013). Indeed, dI3 INs differentiate themselves from other genetically identified populations (e.g. RORalpha, Bourane et al. 2015) by our knowledge of their roles in two distinct but related forms of motor behaviours at two distinct developmental time points, namely the palmar grasp reflex and volitional hand grasp. Therefore, dI3 INs offer an unique opportunity to study the development of circuitry responsible for both the reflex and grasping in adults.

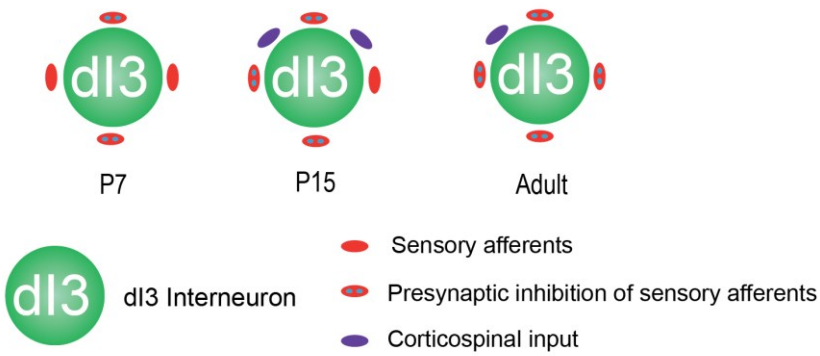

Figure 7. Diagram of the maturation of inputs to dI3 INs throughout development. Qualitative differences in synaptic inputs to dI3 INs are illustrated.

3.1 Disappearance of the PGR

Primitive reflexes, which can be elicited during late fetal development and postnatally (Petrikovsky and Kaplan 1993), are thought to be sets of evolutionary conserved behaviours that promote survival in neonates (Futagi et al. 2012). Although primarily used as diagnostic tools in clinical settings (Capute et al. 1982; Futagi et al. 1992), little is known about how these circuits mature during development and how they mediate additional motor behaviours in the adult nervous system. I show that the PGR begins to disappear around P17 and is completely absent by P21, suggesting that critical developmental changes occur during this time period.

3.2 Development of sensory afferents contacting dI3 INs

I have shown that during development, dI3 INs are contacted by similar numbers of sensory afferents. Our data are in accordance with previous quantifications of sensory afferents contacting dI3 INs (Bui et al. 2013). Although both the CST and sensory afferents were labelled using vGLUT1, the small excitatory contribution of the CST indicates that vGLUT1 labelling provides an overall representative picture of sensory contacts on dI3 INs.

While similar data for other genetically identified INs is not yet available, MNs have been known to undergo substantial sensory pruning during development (Gibson and Clowry 1999; Gibson et al. 2000). However, it is possible that due to their different roles, these different neuronal cell types undergo contrasting sensory refinement or lack thereof.

I had initially hypothesized that to explain the disappearance of the PGR, sensory innervation to dI3 INs might decrease during maturation. I found that as dI3 INs mature, the amount of sensory innervation, determined as the number of terminals, remained constant during

postnatal development. It is important to note that immunohistochemical experiments cannot measure the synaptic activity of these terminals; however, previous electrophysiological experiments have determined that dI3 INs respond similarly to sensory stimulation during early development suggesting that synaptic weight of these terminals remains similar throughout development (Bui et al. 2013).

Although dI3 INs are found in similar densities in the cervical and lumbar segments (Bui et al. 2013), the grasping produced by the forepaws grants greater dexterity whereas the grasping of the hind paws is generally more coarse and is associated with locomotor behaviours. Therefore, I hypothesized that to account for the increased dexterity of forepaw grasping, dI3 INs in cervical as opposed to lumbar segments would receive greater amounts of sensory afferents. I found that dI3 INs in both segments received similar numbers of sensory terminals. Indeed, these data are interesting as they suggest that both spinal segments require similar amounts of sensory information in order to produce two distinct grasping movements. It is worth noting that no study has experimentally determined the relative amounts of sensory afferents contacting the cervical and lumbar segments. In addition, due to the observed small size of varicosities in younger animals, there is a possibility to have underestimated the actual number of sensory terminals present on dI3 INs.

3.3 Presynaptic inhibition of primary afferents contacting dI3 INs

I have successfully shown that primary afferents contacting dI3 INs receive increasing presynaptic inhibitory terminals during development. Indeed, presynaptic inhibition has been shown to be essential in mediating reaching grasping movements in mice (Fink et al. 2014).

In addition, our findings provide novel insights into the development of presynaptic inhibitory terminals in the spinal cord. dI4 INs, which are the largest source of presynaptic inhibition in the spinal cord, have been shown to receive input from the CST (Russ et al. 2013). However, it is currently unknown if the development of the CST to these INs affects the maturation of presynaptic inhibition. Indeed, if CST development were to affect maturation of GABApre terminals, one would expect to see a time lag in the development of presynaptic inhibitory terminals in cervical and lumbar segments. I was able to show that presynaptic inhibitory terminals develops at a similar rate in cervical and lumbar segments suggesting that CST maturation is not strongly linked to development of GABApre terminals onto primary afferents contacting dI3 INs.

Recently, release of BDNF and glutamate from primary afferents has been shown to lead to changes in GAD65 and 67 expression, respectively, in GABApre terminals (Mende et al. 2016). It is possible that levels of BDNF in primary afferents changes during maturation thus increasing production of GAD65 and leading to increased synaptic transmission. Whether expression levels of BDNF in primary afferents change during development remains to be determined but this insight will be essential in determining the involvement of presynaptic inhibition in the loss of the PGR.

Previous experiments have concluded that at birth, GABApre terminals contacting MNs are devoid of associated molecular markers (Betley et al. 2009). I have shown that at P3, roughly 40% of primary afferents contacting dI3 INs were contacted by GABApre terminals. Considering that the development of presynaptic inhibitory terminals is similar for both the

dorsal and ventral horn, the first few days of development seem to be essential for the development of presynaptic terminals. However, it is possible that development of dI4 INs progresses differently in the dorsal and ventral horn.

3.4 Maturation of motor control

Many of our skilled movements are controlled by the corticospinal tract and grasping is no different. Indeed, lesions studies have implicated the CST in grasping in the rat (Alstermark and Isa 2012). Our data, in accordance with previous experiments, demonstrated that dI3 INs received reduced CST input as development progressed. In parallel, I determined that the number of sensory afferents contacting dI3 INs remained constant throughout development. Therefore, it is tempting to hypothesize that in response to diminishing CST input, the number of sensory afferents contacting dI3 INs might increase. Indeed, competition between sensory afferents and CST inputs has been shown to produce sprouting/elimination of competing fibers (Jiang et al. 2016). However, additional experiments comparing the number of both sensory afferents and CST input on individual dI3 INs will be necessary to form a definitive conclusion. Primitive reflexes are presumed to disappear in response to the increasing cortical inhibition. Considering that presynaptic inhibition is likely not the sole factor responsible for the loss of the PGR, dI3 INs presumably receive additional pre- or postsynaptic inhibition during development.

3.5 Mechanisms of development of motor circuits

Although I have quantified sensory afferents and the corresponding presynaptic terminals contacting dI3 INs, it is possible that the functionally or synaptic weight of these connections varies during development. Indeed, although immunohistochemistry is a powerful technique, it does not capture the functionally of synapses. Functional changes in synapses involve many factors such as channel composition, channel expression and quantity of NT released to name just a few.

Autoradiographic binding studies have suggested that expression of the glutamate activated NMDA channel decreases in the dorsal horn during maturation (Gonzalez et al. 1993). Further studies have determined that binding to NMDA receptors in spinal motor nuclei decreases during development (Verhovshek et al. 2005). Similarly, changes in expression of NMDA subunits has been linked with development (Watanabe et al. 1994). In particular, expression of the GluN3A subunit was found to peak at the end of the first postnatal week (Ciabarra et al. 1995). In addition, recent studies indicate that presynaptic expression of the GluN3A subunit may be linked to glutamate release (Larsen et al. 2011).Therefore, it is conceivable that these changes alter the synaptic weight of glutamate releasing fibers onto dI3 INs.

In addition to varying strengths of inputs to dI3 INs, it is possible that their intrinsic properties are modified during development such that they become more or less excitable, and this would have important consequences on the integration of synaptic inputs to dI3 INs. Indeed, input resistance, I-V relationship and specific membrane resistance of neurons has been known

to change during development (McCormick and Prince 1987). Therefore, it is possible that changes in electrophysiological properties of dI3 INs alter their excitability during development.

Intrinsic properties affect synaptic integration but also firing properties of neurons. Indeed, previous electrophysiological experiments have determined that dI3 INs respond to stimulus with three types of firing behaviours: tonic firing, initial bursting and delayed firing (Bui et al. 2013). However, how these behaviours evolve during development and how they alter the activity of the network remains to be determined. Alternatively, it is important to note that the process of myelination is not yet complete at birth and is ongoing until the second week of maturation (Webster 1971). Indeed, myelination of fibers can be necessary in order to summate several weaker signals into an appropriate output response. Whether myelination of certain supraspinal and sensory fibers is implicated in the development of dI3 INs remains to be determined.

In addition to being shaped during development, intrinsic properties can be modulated by neuromodulators such as monoamines (dopamine, serotonin, noradrenaline, etc.). Neuromodulators can modify the AP threshold, I_h and persistent inward currents to name just a few (Zhong et al. 2006; Dai and Jordan 2010a, 2010b). Specifically in spinal interneurons, serotonin has been shown to modulate synaptic transmission as well as depolarize the resting membrane potential (Hammar et al. 2004; Carlin 2005). Additionally, noradrenaline has been known to increase excitability of spinal interneurons via activation of α1-adrenoreceptor (Elliot and Wallis 1992). Spinal circuits are believed to be influenced by neuromodulators. For example, central pattern generator (CPG) INs involved in locomotion have been known to receive

modulatory monoaminergic input from dopaminergic, serotonergic and noradrenaline fibers (Jordan et al. 2008; Miles and Sillar 2011) and the activity of CPGs may require the presence of these neuromodulatory inputs. In addition, serotonergic input has been linked to circuit assembly of these networks during development (Okado et al. 1992). Recent research has determined that dI3 INs possess an accessory role in locomotion and therefore it is possible that they receive some monoaminergic input during development (Bui et al. 2016). Hence, it is conceivable that the development of dI3 IN function is dependent upon the development of neuromodulatory inputs through postnatal stages (Vinay et al. 2002; Miles and Sillar 2011).

Although dI3 INs were found to receive CST input, I suspect that during development, dI4 INs receive increasing CST innervation. Given the importance of presynaptic inhibition in generating smooth movements (Fink et al. 2014), it is critical for the CST to be able to silence innocuous sensory information during volitional grasping. Additionally, I suspect that CST inputs to dI4 INs likely serves as the main excitatory drive for presynaptic inhibition of primary afferents contacting dI3 INs.

3.6 A model of dI3 IN circuitry and operation in the adult nervous system

In summary, I believe dI3 INs to be essential sensorimotor centers of the spinal cord being able to integrate sensory, supra and spinal information into the production of grasping (Figure 8). Indeed, due to their CST input, dI3 INs appear to serve as more than simple sensorimotor integration centers. During volitional grasping, I suggest that the CST provides the main excitatory drive to dI4 INs and thus maintains presynaptic inhibition of primary afferents contacting dI3 INs. This prevents sensory afferents, particularly those providing sensory

feedback from the hand, to activate dI3 INs and to excite motoneurons involved in grasping in response to any and all tactile stimuli. In addition to the CST control of presynaptic inhibition that I propose, I found only a small number of CST terminals onto dI3 INs. Therefore, supraspinal excitation of dI3 INs, if any, must come from another descending pathway. The reticulospinal tract (ReST) originates from the reticular formation in the brainstem and has been shown to be implicated in hand movements in primates (Riddle and Baker 2010). Therefore, I suggest that the Reticulospinal tract (ReST) instead of the CST provides the main supraspinal excitatory drive. Indeed, recent lesion studies have demonstrated that the ReST may have a larger contribution then the CST towards grasping (Alstermark and Pettersson 2014), therefore serving as likely source of excitation to dI3 INs. The CST could still have an excitatory influence over dI3 INs through excitation of the ReST. Although, there are currently no immunohistochemical markers for ReST input, electrophysiological and labelling methods have been successful in implicating the ReST in spinal circuits (Bretzner and Brownstone 2013; Lenschow et al. 2016). Alternatively, I suggest that during cutaneously evoked reflexive grasping, sensory afferents provide the sole excitatory drive to dI3 INs. In order to prevent constant excitation by any innocuous stimuli to the hand, dI3 INs are likely tuned to specific sensations such as that of a slipping object or rapidly increasing loads. Specialized receptors in the hand such as Merkel cells and Ruffini corpuscles convey the information of slippage (Johansson and Flanagan 2009), and dI3 INs may receive prominent sensory feedback from sensory neurons innervating these receptors. It is now possible to confirm that dI3 INs can mediate both cutaneously evoked grasping via sensory afferents as well as voluntary grasping via the CST in the adult through direct synaptic contacts by the respective pathways, though it remains to be seen whether the CST or ReST have greater excitatory sway over dI3 INs.

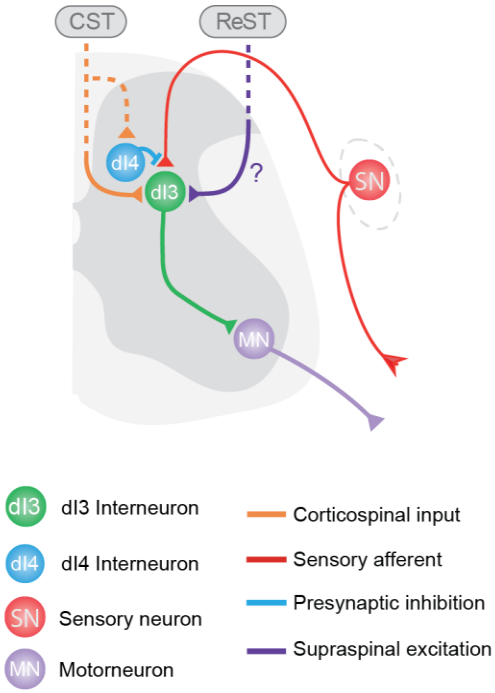

Figure 8. Diagram of the putative connectivity of dI3 INs involved in control of hand grasp

Schematic showing the relationship of dI3 INs (green) to dI4 INs (teal), corticospinal input (orange), sensory input (SN, red), motoneurons (MN, light purple), and putative reticulospinal input (dark purple).

3.7 Future directions

This study has determined the developmental time course of the primitive palmar grasp reflex. Further, it has been demonstrated that development of sensory afferents, in terms of number of synapses, onto dI3 INs of cervical and lumbar segments remains constant during development. Further, these sensory afferents were found to receive increasing presynaptic inhibitory terminals during maturation; however, the developmental time course of this inhibition does not appear to match the disappearance of the PGR. Finally, dI3 INs were found to receive corticospinal input and this input was shown to decrease during development.

Future studies should focus on studying the developmental implications of supra- and spinal inhibition to dI3 INs. Ptf1a labelled dI4 INs are the main source of presynaptic inhibition to primary afferents in the spinal cord (Betley et al. 2009). In light of this, studying the development of CST contacts onto GABApre neurons would provide valuable insights into how supraspinal centers modulate the flow of sensory information to spinal interneurons involved in grasping. Given that prior studies of both dI3 and dI4 INs make use of conditional expression of CRE, future studies of the development of descending control of presynaptic inhibition specific to sensory inputs to dI3 INs may rely upon intersectional genetic strategies involving crossing one of the CRE driver line with a newly generated FLP-FRT or I-SceI construct of the other interneuron population. Although methodologically demanding, these experiments would afford the novel ability to monitor the development of dI3 INs in parallel to dI4 INs and CST input. In addition, electrophysiological experiments where presynaptic inhibition is elicited by stimulation of adjacent dorsal roots in conjunction with stimulation of the CST will offer novel insights about how supraspinal centers modulate sensory transmission to dI3 INs.

Recently, V2a INs were found to contain a population of excitatory propriospinal neurons (PNs) projecting to the LRN, a pre-cerebellar relay center believed to receive internal copies of pre-motor signals which are relayed back to the PNs through the ReST(Azim et al. 2014). Subsequent ablation of V2a INs led to impairments in reaching behaviours thus confirming their involvement in a cerebellar feedback loop (Azim et al. 2014). In light of these results and with the knowledge that dI3 INs project to the LRN, future experiments should focus on determining if the ReST contacts motor pools involved in grasping.

Among the excitatory extrapyramidal tracts, the ReST distinguishes itself by containing a subset of inhibitory fibers (Du Beau et al. 2012). In addition to being involved in grasping (Alstermark and Pettersson 2014), ReST fibers have been found to contact excitatory and inhibitory spinal interneurons (Esposito et al. 2014). Altogether, these findings make the ReST an ideal candidate for putative direct excitation and inhibition to dI3 INs. The expression of Lhx3 and/or Chx10 specifies a population of reticulospinal glutamatergic neurons (Bretzner and Brownstone 2013). However, as described above, these studies would require the generation of new genetic constructs.

To understand how dI3 INs are activated during sensory-evoked increases in grip strength, it may be important to map the receptive field of individual dI3 INs. This will provide insights into the sensory stimulation that activates these neurons, and by extension, leads to increase in grip strength. Alternatively, to further understand how dI3 INs coordinate grasping, it will be necessary to identify the degree at which dI3 INs innervates the different motor pools associated with grasping. These experiments would entail injecting anterograde tracers in muscles of interest and determining if the overlap with dI3 INs on select MNs. Indeed, dI3 INs

place themselves as an essential element of the spinal circuitry involved in grasping and furthering our understanding of their development will yield great insights about how a particular class of spinal interneurons mediates grasping.

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Appendices

Appendix 1

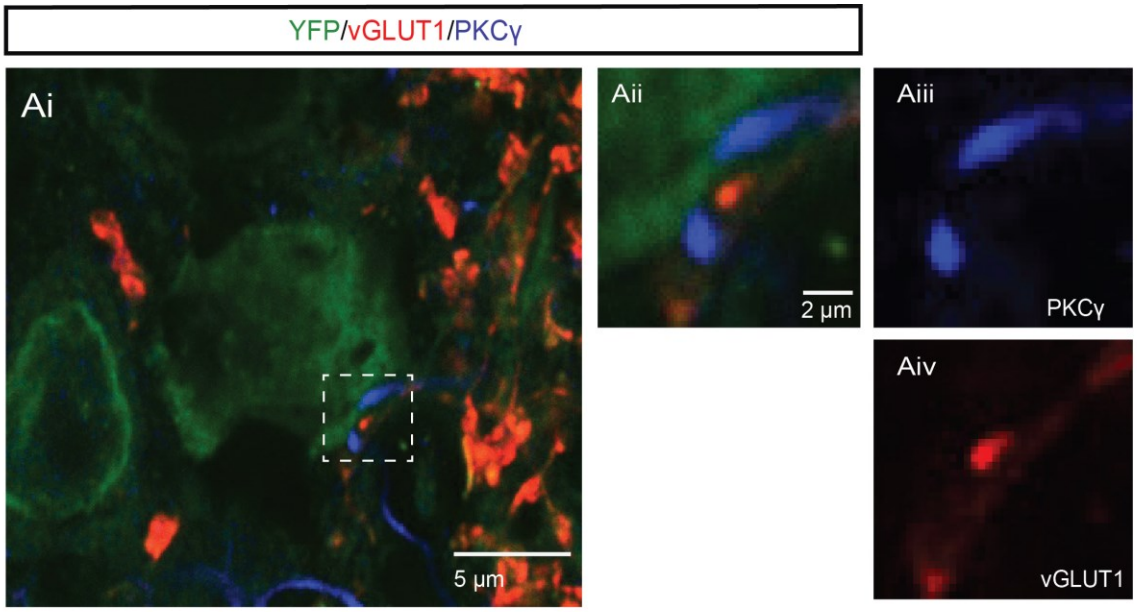

Figure S1. dI3 INs receive PKCγ⁺/vGLUT⁻ input

(A) PKCγ⁺/vGLUT⁻ terminals contact a cervical dI3 IN from a P14 spinal cord. Bouton in the dashed box is magnified in (Aii)-(Aiv).

Appendix 2

Table S1. Comparisons of the incidence of presynaptic inhibition of primary afferents contacting dI3 INs among age groups in cervical and lumbar segments.

Age groups	Significant	Adjusted P Value
Cervical		
P3 vs. 7	Yes	0.0017
P3 vs. 11	Yes	0.0008
P3 vs. 15	Yes	<0.0001
P3 vs. 60	Yes	<0.0001
P5 vs. 7	Yes	0.0263
P5 vs. 11	Yes	0.0150
P5 vs. 15	Yes	0.0002
P5 vs. 60	Yes	<0.0001
P3 vs. 7	Yes	0.0017
Lumbar		
P3 vs. 15	Yes	<0.0001
P3 vs. 60	Yes	<0.0001
P5 vs. 7	Yes	0.0030
P5 vs. 11	Yes	0.0007
P5 vs. 15	Yes	<0.0001
P5 vs. 60	Yes	<0.0001
P7 vs. 15	Yes	0.0438
P7 vs. 60	Yes	0.0010
P11 vs. 60	Yes	0.0013