University of Alberta

The V0 Interneurons: First-Order Interneurons of the Locomotor CPG?

by

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Abstract

The locomotor Central Pattern Generator (CPG) is a neuronal network capable of producing rhythmic locomotor output independent of sensory or descending input. Attempts to identify component interneurons of the CPG have been aided by the discovery of transcription factors that are expressed by discrete interneuron populations during development. The V0 interneuron population is defined by the expression of the transcription factor Dbx1. Herein I test the hypothesis that V0 interneurons are first-order cells of the locomotor CPG responsible for initiating rhythmic locomotor activity. Anatomical tracing from brainstem sites known to be responsible for the initiation of locomotion reveal that these regions make monosynaptic connections onto V0 cells. Immunohistochemistry demonstrates that V0 cells express 5-HT_7 and 5-HT_{2A} receptors, previously shown to be required for locomotor initiation. Taken together these results support the hypothesis that V0 interneurons are first-order interneurons of the locomotor CPG responsible for initiating locomotion.

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Part I: Early experiments and basic principles of locomotion.

Vertebrates engage in rhythmic movements which include walking, swimming, breathing, and chewing. The appropriate patterns of muscle activation required for these behaviours are generated by neuronal networks present in the central nervous system (CNS), termed Central Pattern Generators (CPGs). One of the defining features of CPG circuits is that they are capable of producing rhythmic output in the absence of input from sensory organs or descending input from higher brain centres. While the term "Central Pattern Generator" first arose in the literature in the mid-1900's (Wilson and Wyman, 1965), evidence for the existence of a vertebrate locomotor CPG, the network of neurons in the spinal cord that is responsible for generating and coordinating rhythmic locomotor activity, was first demonstrated early in the 20th century (Brown 1911, 1914). Prior to Brown's hypothesis, physiologists had performed lesions of the cerebral cortex in birds and dogs in order to investigate its various functions in perception and behaviour (Unzer, 1771; Flourens, 1824). They found that the primary motor cortex, the region of the cerebral cortex which evokes discrete movements when stimulated electrically (Fritsch and Hitzig, 1870), was not required for fully functional locomotion in dogs and birds since this behaviour persisted following transection of the CNS just rostral to the midbrain (Flourens, 1824). Furthermore, Freusberg (1874) demonstrated functional postural weight support as well as the induction of stepping movements in the hindlimbs after complete thoracic spinal cord transection. The observation that mammalian locomotion can occur

independent of any input from the cerebral cortex led to a flurry of experiments and theories in the late 19th and early 20th centuries aimed at identifying the mechanisms by which locomotor movements are generated.

Early theories to account for locomotor behaviour following decerebration or spinalization placed much emphasis on the role of sensory afferents and reflex pathways in the control of stepping patterns. Among these early theorists was Maurice Philippson, who studied stepping movements and reflexes in chronically maintained spinalized dogs. Philippson (1905) provided a detailed description of the step cycle and the timings of reflexes which he believed to play a critical role in the control of stepping movements. He postulated that locomotor control could be attributed to a combination of central and reflex mechanisms (for review see Clarac, 2008; Stuart, 2008).

Charles Sherrington (1910) studied reflex movements of the limbs in response to cutaneous and proprioceptive sensory stimuli. In particular, Sherrington focused his studies on the flexion-reflex, in which a painful stimulus applied to the skin of the foot or toe triggers the activation of ipsilateral flexor muscles, resulting in the withdrawal of the leg (Sherrington, 1910), and the crossed extension-reflex, which occurs in conjunction with the flexion-reflex and results in the activation of extensor muscles in the contralateral leg providing weight support. Sherrington observed that these reflex movements bear similarities to the alternation of contralateral flexor and extensor muscles that occurrs during locomotion, and observed reflex “stepping” in the decerebrate cat and dog (Sherrington, 1910). He theorized that locomotor rhythms are generated

by reflexive stepping, and that alternation is enforced by reflex "fatigue," in conjunction with static reflexive maintenance of posture (Sherrington, 1910).

Sherrington's theories on reflexive locomotion were countered by the experiments of one of his own trainees, Thomas Graham Brown, who studied spontaneous locomotor-like movements that occur in the cat while under deep anesthesia, a phenomenon he termed "narcosis progression". Graham Brown (1911, 1914) electrically stimulated the primary afferent nerves and observed that while the peripheral reflexes are abolished by the chemical narcotic, "narcosis progression" may still occur. Based on these observations, Graham Brown believed the basis of locomotor control to be primarily central rather than peripheral. Using his "narcosis progression" preparation, Graham Brown tested his hypothesis that peripheral reflexes are not required for the generation of locomotion by demonstrating that rhythmic, alternating, locomotor-like movements can be elicited in the hindlimbs of the cat after both complete thoracic spinal cord transection and complete hindlimb sensory deafferentation by destruction of the dorsal roots (Graham Brown, 1911, 1914). This indicates that all of the circuitry required for the generation of the basic locomotor rhythm is contained entirely within the spinal cord. In these same studies he demonstrated that stepping movements persist in one limb after midsagittal division and removal of the contralateral half of the spinal cord, indicating that each limb is controlled by centres that are localized in the ipsilateral half of the spinal cord. In a later paper, Graham Brown (1915) observed both spontaneous and asphyxia-induced rhythmic locomotor-like movements in a decerebrate cat fetus that had

been extracted and immersed in physiological solution, indicating that central neural control of locomotion is an innate rather than a learned function of the CNS.

Graham Brown (1914) also posited a theory as to the mechanism by which the locomotor CPG produces rhythmic alternating activation of leg flexor muscles (involved in the swing phase of locomotion) and extensor muscles (involved in the stance phase of locomotion), his "half-centre" hypothesis. Graham Brown's original model was relatively simple with the motoneurons innervating the flexor muscles comprising one half-centre and the motoneurons innervating the extensor muscles comprising the second half-centre (**Figure 1**). Graham Brown (1914) suggested that these half-centres provide reciprocal inhibition to one another directly via inhibitory branches, although he did mention the possibility of interposed neurons in this arc. He further suggested that this inhibition is conditioned by a temporal process of "depreciation of inhibition," perhaps through a mechanism of fatigue which triggers the switch in the step cycle between flexor and extensor bursting. While it has become clear that the true CPG circuitry is much more complicated than this, with rhythmic excitatory drive to motoneurons as well as reciprocal inhibition being provided by CPG interneurons, Graham Brown's conception of reciprocal inhibition and his simplified "half-centre" model have proven influential, and have been embraced and expanded upon by subsequent generations of neuroscientists.

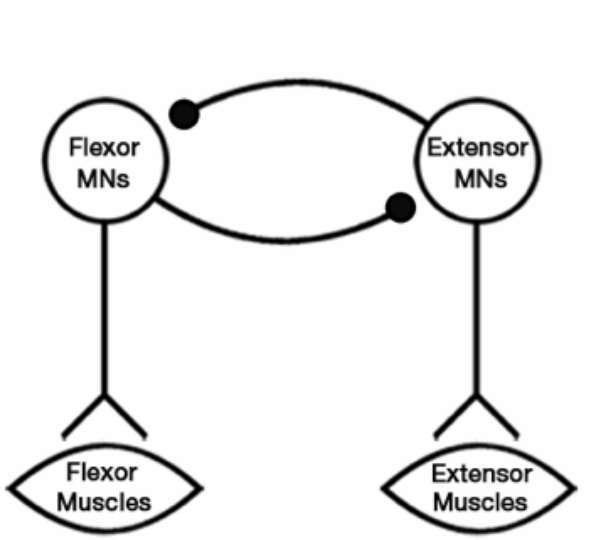

Figure 1: Thomas Graham Brown's original "Half-Centre" hypothesis. Graham Brown's (1914) original "half-centre" model for the mechanism of central generation of locomotor rhythms. The efferent motoneurons innervating the flexor muscles comprise one "half-centre" (Flexor MNs) and the efferent motoneurons innervating the extensor muscles comprise the second "half-centre" (Extensor MNs). At the time Graham Brown (falsely) theorized that the motor neurons themselves, although principally excitatory in nature, possessed inhibitory branches which were responsible for reciprocal inhibition between half-centres.

Two key developments in the late 1940's and early 1950's would change the shape of the field of neuroscience, and revolutionize central pattern generator research. Prior to this time, the microscopic size of the mammalian neuron limited researchers' capacity to record from and evaluate the electrical activity of individual neurons. This problem was circumvented by Hodgkin and Huxley, who utilized the axial wire technique of Marmot (1949) to make intracellular recordings from the giant axon of *Loligo* (squid), and subsequently developed the voltage clamp technique in order to study and model the current-voltage relationships and changes in membrane conductance that occur during the generation of an action potential (Hodgkin *et al.* 1952; Hodgkin and Huxley, 1952). The mathematical descriptions of the action potential developed by Hodgkin and Huxley are still in use today. Almost concurrently, the invention of the glass capillary microelectrode and its use for intracellular recording by Ling and Gerard (1949) allowed researchers to make intracellular recordings from individual neurons in mammalian spinal cord tissue.

In the following decades, the field of locomotor research was largely dominated by *in vivo* electrophysiological experiments in the cat. Shik *et al.* (1966a,b) described a method for the generation of coordinated treadmill locomotion in the decerebrate cat *in vivo* by electrical stimulation of a distinct location in the midbrain, now known as the Mesencephalic Locomotor Region (MLR). Modulating the intensity of the MLR stimulation directly affected the speed of locomotion produced, and at high speeds the animals automatically switched from a "trotting" (left-right alternation) to a "galloping" (left-right

synchrony) gait (Shik *et al.* 1966a). Alternatively, if the intensity of MLR stimulation was kept constant but the speed or angle of the treadmill was modulated, the animals automatically adapted running speed, and force produced to accommodate the environmental changes (Shik *et al.* 1966a). A number of segmental reflex pathways were evaluated during MLR stimulation, and it was found that these reflexes are modulated in such a direction as to promote optimal efficiency of work of the muscles in the stepping leg (Shik *et al.* 1966b).

Anders Lundberg and his colleagues, including Elzbieta Jankowska, played a critical role in the refinement of Graham Brown's half-centre model for locomotor rhythm generation. Much of Lundberg's most significant work was based on the development and utilization of intracellular recording techniques to record from spinal cord neurons in the cat *in vivo* (Andén *et al*. 1966a,b; Jankowska *et al.*1967a,b). Lundberg and his colleagues were following up on work by Carlsson *et al.* (1963) showing that L-DOPA and 5-HTP significantly increase the flexor reflex in the acute spinal cat; Lundberg went on to analyze the effects of L-DOPA on flexor reflex pathways using intracellular recordings from motorneurons and interneurons in the spinal cord (Jankowska *et al.* 1967a,b). When combined with stimulation of sensory afferents (Grillner, 1969), or coadministered with the monoamine oxidase inhibitor Nialamide (Jankowska *et al.* 1967b; Grillner and Zangger, 1979), intravenous L-DOPA evokes spontaneous, rhythmic, alternating flexor and extensor motorneuron activity. Recordings from motoneurons during flexor reflex stimulation under L-DOPA revealed the strong mutual inhibitory interactions between flexor and extensor

motorneuron pools (Jankowska *et al.* 1967a) that had been hypothesized to exist (Graham Brown 1914). Following L-DOPA administration, activation of the flexion reflex revealed interneuron pools that were rhythmically active and appeared to also display strong mutual inhibitory interactions (Jankowska *et al.* 1967b), leading Lundberg and his colleagues to believe that the CPG half-centres are actually interneuronal in nature rather than being comprised of the motorneurons themselves. These interneuron "half-centres" were proposed to provide rhythmic excitatory input to their respective (ie. flexor) motoneuron pools, and rhythmic inhibitory input to their opposing (ie. extensor) motoneuron pools, as well as reciprocal inhibition between flexor and extensor interneuron half-centres (**Figure 2**; Jankowska *et al.* 1967a,b; Lundberg, 1981).

Lundberg and his colleagues went on to describe in detail the convergence of descending inputs from the brainstem reticular formation and ascending sensory inputs onto interneurons in the spinal cord via an indirect technique in which motorneuron activity was monitored while each of the convergent pathways are given a subthreshold stimulus. Each individual stimulus produced no response in the motoneuron, however when applied in close succession to enable summation, the integration of both signals activated the motorneuron (Jankowska and Lundberg, 1981; Illert *et al.* 1981; Lundberg, 1981). By demonstrating the convergence of descending locomotor command systems and sensory feedback signals onto interneurons, they identified a population of interneurons that is an attractive cellular substrate for these half-centre CPG elements (Jankowska and Lundberg, 1981; Illert *et al.* 1981; Lundberg, 1981).

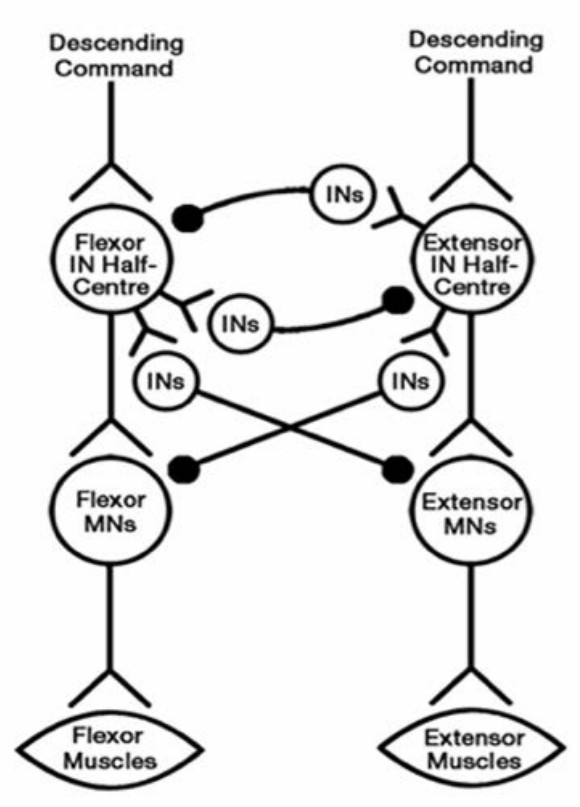

Figure 2: Lundberg's modified "Half-Centre" model. Interneuron (IN) "half-centres" (Flexor IN Half-Centre and Extensor IN Half-Centre above) provide rhythmic excitatory input to their respective motor neuron pools (Flexor MNs and Extensor MNs) as well as reciprocal inhibition (INs) between flexor and extensor interneuron "half-centres" (Jankowska and Lundberg, 1981).

Another theory proposed to explain the generation of locomotor rhythms, which again expanded on the basic principles of Graham Brown's half-centre hypothesis, is the "unit burst generator" theory proposed by Grillner (1981) (**Figure 3**). According to Grillner's theory, which was based on his observations in the lamprey, the CPG is made up of a series of unit burst generators (UBGs) each of which is capable of producing rhythmic activity in a closely related muscle group that acts on a given joint. Such an organization would involve dispersal of such UBGs throughout the cord, and communication between them, as well as integration of information from reflex/sensory systems and higher centres, resulting in the complex patterns of activity observed during locomotion (Grillner, 1981). Investigations of the simple swimming CPG's of "lower" animals, such as Grillner's studies in the lamprey, have provided much information regarding the organization and function of their respective CPG systems, although it is unclear to what extent this information can be applied to the mammalian quadrapedal locomotor CPG.

Graham Brown's "half-centre" hypothesis was recently further expanded upon by McCrea and colleagues, who used the cat *in vivo* fictive locomotor preparation to investigate spontaneous deletions of motorneuron bursting that occur periodically during otherwise normal locomotion (Lafreniere-Roula and McCrea, 2005; Rybak *et al.* 2006a, 2006b; McCrea and Rybak, 2008). These spontaneous deletions are characterized by the coordinated loss of activity in multiple synergist motorneuron pools together with tonic activity in multiple pools of antagonist motorneurons (Lafreniere-Roula and McCrea, 2005; Rybak *et*

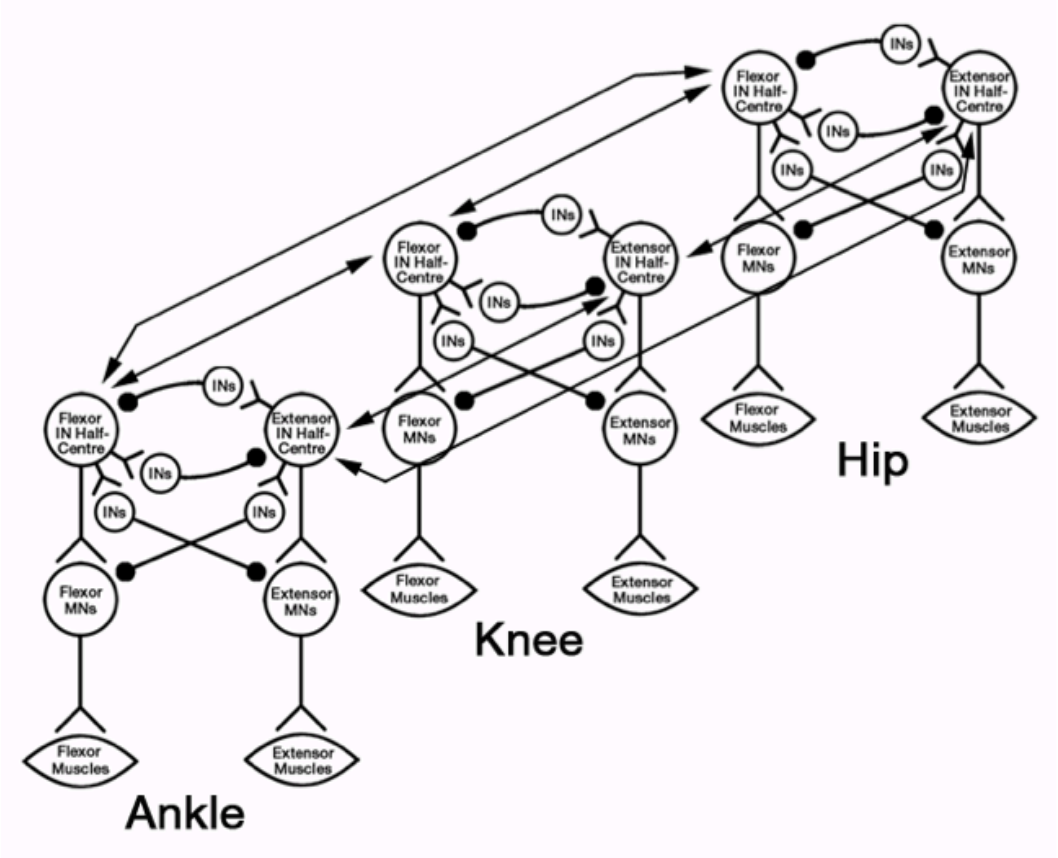

Figure 3: Grillner's "Unit Burst Generator" Hypothesis. According to Grillner's model, the locomotor CPG is made up of a series of "Unit Burst Generators" (UBGs) each of which drives the antagonist muscles that act on a given joint. Each UBG is capable of producing rhythmic locomotor bursting on its own, and coordination is provided by crosstalk between UBGs. (Grillner, 1981).

al. 2006a, 2006b; McCrea and Rybak, 2008). The high level of coordination observed suggests that rather than being independent events, these deletions are likely the result of a loss of activity in an organized and tightly regulated neuronal network that provides input to all motorneurons (McCrea and Rybak, 2008). McCrea and his colleagues observed that after a deletion, the phase and period of locomotor activity was often identical to that before the deletion, and that rhythmic activity often returned after a measure of time that is an integer of the period of the initial rhythm, these have thus been termed 'non-resetting' deletions (Lafreniere-Roula and McCrea, 2005). While 'non-resetting' deletions in fictive locomotor activity had been previously described, it had been postulated that because locomotion is a bilateral behaviour, the maintenance of the rhythm could be due to maintained activation coming from the contralateral side of the CPG. McCrea and colleagues made the observation that 'non-resetting' deletions also occur in fictive scratching, a unilateral behaviour, and based on this observation they ruled out the possibility that activity is maintained by activation coming from the contralateral CPG and suggested that non-resetting deletions can only be explained by the existence of a separate neuronal network which is capable of maintaining the locomotor rhythm while activity is lost at the level of motorneuron activation (Lafreniere-Roula and McCrea, 2005; McCrea and Rybak, 2008). Thus they have suggested a two-level CPG (**Figure 4)** which consists of two separate neuronal networks: a 'pattern formation' (PF) network which exists at the level of motorneuron recruitment and is responsible for the coordination of synergist vs. antagonist muscle groups (activity is lost in the PF

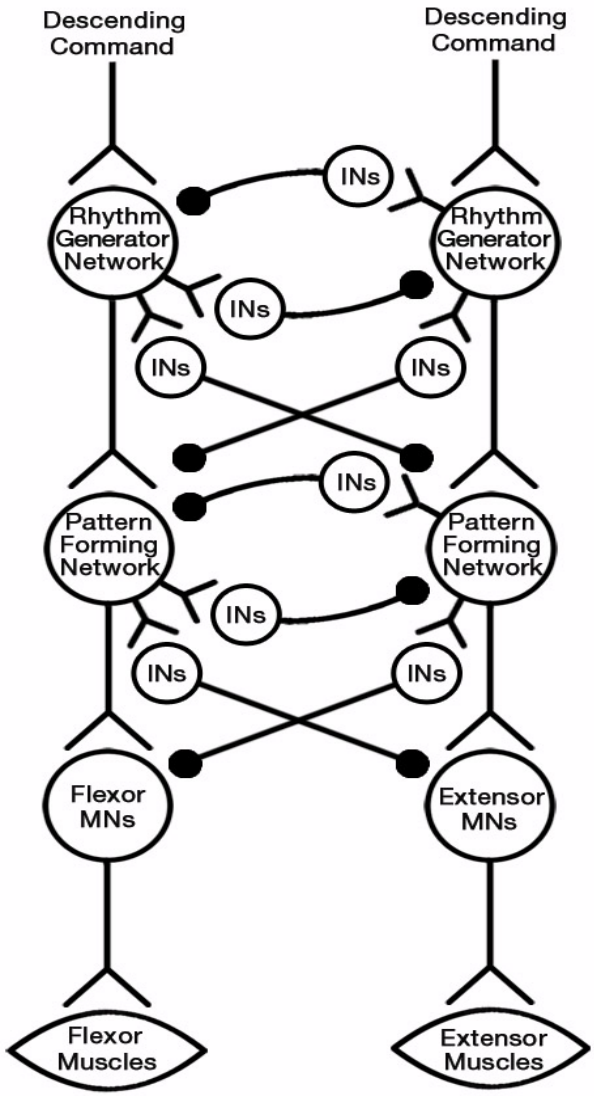

Figure 4: McCrea and Rybak's Two-Level CPG model. 'Non-resetting' deletions of fictive locomotor activity occur in which motor neuron bursting resumes at the same phase and period as before the deletion, and after a unit of time equal to an integer multiple of the original cycle period. In order to explain these 'non-resetting' deletions, McCrea and Rybak (2008) suggested a two-level CPG that consists of a 'Rhythm Generator' (RG) network which is responsible for generating the rhythm and which drives a second CPG network, the 'Pattern Formation' (PF) network, which is responsible for motor neuron recruitment and the precise coordination of synergist vs. antagonist motor neuron activation. Activity of the 'Pattern Formation' network is thought to be specifically silenced during 'non-resetting' deletions while activity of the 'Rhythm Generator' network persists, resulting in the maintenance the phase and period of bursting when locomotor activity resumes.

network during non-resetting deletions), and a ‘rhythm generator’ (RG) network which exists entirely at the interneuronal level and is responsible for the generation of the locomotor rhythm as well as the timing of the phase and period of oscillation (activity persists in the RG network during non-resetting deletions) (Rybak *et al.* 2006a, 2006b; McCrea and Rybak, 2008). The RG network could therefore be thought of as the CPG ‘clock’ which drives rhythmic activity in the PF network, which in turn activates the appropriate host of motorneurons resulting in coordinated locomotor activity (McCrea and Rybak, 2008).

Part II: The *in vitro* fictive locomotor preparation.

The development of an *in vitro* preparation for the study of the mammalian locomotor CPG has been revolutionary. The original *in vitro* experiments were performed on spinal cords dissected out from neonatal rats with the hindlimbs attached. Bath-applied NMDA (N-methyl-D-aspartate) induced locomotor rhythms that could be monitored via electromyogram (EMG) electrodes placed on the hindlimb muscles (Kudo and Yamada, 1987; Smith and Feldman, 1987). The preparation more commonly used today involves the co-application of 5-HT (Serotonin) and NMDA to the isolated spinal cord to evoke a very robust rhythm that is recorded via suction-electrodes placed on the ventral roots of the lumbar spinal cord (Kjaerulff and Kiehn, 1996; Butt et al. 2002). Rhythmic, alternating activity can be observed between ipsilateral L2 ventral roots (which predominantly consist of motor axons innervating hindlimb flexor muscles) and L5 ventral roots (which predominantly consist of motor axons innervating hindlimb extensor muscles). Left-right alternation between contralateral flexor related, as well as contralateral extensor, related ventral roots, is also observed. (Kjaerulff and Kiehn, 1996; Butt et al. 2002).

This preparation was used to determine the specific regions in which the locomotor CPG is located. Kjaerulff and Kiehn (1996) monitored locomotor activity via ventral root recordings while performing mechanical lesions of the spinal cord in order to determine which regions are most important for the generation of strong, rhythmic, coordinated bursting. Based on their findings, it has become clear that spinal locomotor networks are localized ventromedially, as

the ventral third of the spinal cord is capable of producing rhythmic activity when isolated, while the lateral regions are not (Kjaerulff and Kiehn, 1996). This same study demonstrated that rhythm-generating networks are distributed throughout the lumbar region of the spinal cord, and are also present in the lower thoracic region, with rhythmogenic capacity distributed in a rostral-to-caudal gradient (Kjaerulff and Kiehn, 1996). They were also able to localize crossed connections responsible for the coordination of left-right alternation to the ventral commissure. These crossed connections are distributed throughout the entire lumbar spinal cord, with control of left-right alternation distributed in a rostral-to-caudal gradient, as it was observed that lesions of the ventral commissure in the caudal lumbar cord do not affect left-right alternation if crossed connections in the rostral lumbar cord are left intact (Kjaerulff and Kiehn, 1996). Finally, they found that connections responsible for coordination along the rostral-caudal axis (i.e. rhythmic alternation of L2 and L5 ventral roots) are distributed throughout both the lateral funiculus and the ventral funiculus, as either of these tracts are sufficient for alternation of L2 and L5 roots when the other is lesioned, but lesioning of both funiculi disrupts coordinated ipsilateral L2-L5 alternation (Kjaerulff and Kiehn, 1996).

The *in vitro* fictive locomotor preparation has also provided researchers with a much-improved ability to investigate the pharmacological nature of the locomotor CPG, by simply adjusting the solution in which the isolated spinal cord is located. Cowley and Schmidt (1994; 1997) were among the first to investigate the effects of various neurotransmitters on CPG activity. They found that

application of 5-HT alone is sufficient to elicit rhythmic locomotor activity from the isolated lumbar spinal cord, although the bursting is much slower and less robust than that elicited by the combination of 5-HT and NMDA (Cowley and Schmidt, 1994; 1997). Application of either NMDA, or acetylcholine (ACh), alone, elicited rhythmic bursting, but the patterns of ventral root activity were described as "non-locomotor-like" with common occurrences of co-bursting between ipsilateral flexor and extensor motor pools (Cowley and Schmidt, 1994; 1997). NMDA or Ach was sufficient to elicit bursting activity even in isolated hemisegments (Cowley and Schmidt, 1997). 5-HT on the other hand, was only effective in eliciting rhythmic activity in the intact cord; specifically, an intact connection between the upper lumbar (L1-L2) and lower thoracic (T12 minimum) segments was required (Cowley and Schmidt, 1997). In the context of the two-level locomotor CPG architecture put forward by Rybak and McCrea (2006a; 2006b; described above), it appears that NMDA or Ach may activate a rhythm generator (RG) network whose machinery is distributed through all levels of the spinal cord, but without recruitment of the pattern formation (PF) network, thereby producing rhythmic bursting without locomotor-like coordination. In turn, it appears that 5-HT is responsible for the recruitment/activation of the pattern formation (PF) network, critical elements of which are localized to supralumbar and upper lumbar segments, thus signaling the commencement of the coordinated motor activity known as "walking".

It is also interesting to consider the role of the inhibitory neurotransmitters γ-aminobutyric acid (GABA) and glycine during locomotor activity. During 5-

HT/NMDA evoked fictive locomotion, application of the $GABA_A$ receptor agonist muscimol, the $GABA_B$ receptor agonist baclofen, or glycine, abolishes the locomotor activity (Cowley and Schmidt, 1995). Alternatively, application of the $GABA_A$ receptor antagonist bicuculline or the glycine receptor antagonist strychnine has the effect of converting rhythmic, alternating activity to rhythmic, synchronous activity (Cowley and Schmidt, 1995; 1997). Based on the occurrence of synchrony rather than simply a loss of alternation, this result reveals not only the critical importance of both GABAergic and glycinergic inhibition for normal alternation during locomotion, but also the existence of reciprocal excitatory connections between locomotor CPG interneuron pools (both between flexor and extensor "half-centres" and also commissurally), which are likely silenced by overwhelming reciprocal inhibitory interactions during normal locomotion (Cowley and Schmidt, 1997). The potential strength of these reciprocal excitatory connections is illustrated by the fact that bilateral synchrony between all flexor and extensor motoneuron pools persisted despite extensive sagittal lesions that left commissural connections intact in only one or two segments, at any level on the rostrocaudal axis (Cowley and Schmidt, 1997). These reciprocal excitatory connections between CPG networks could serve as a mechanism for synchronous activation of ipsilateral flexor and extensor muscles in order to increase tone during locomotion. In quadrupeds, these connections could also be responsible for the switch from a walking to a galloping gait.

The role of inhibitory interneurons in locomotor rhythm generation was further examined pharmacologically by Kjaerulff and Kiehn (1997), who utilized

a novel split bath preparation in which the left and right sides of the cord can be exposed to entirely separate bathing solutions. Application of 5-HT and NMDA to one side of the cord induced rhythmic locomotor activity on that side only, illustrating that commissural connections are insufficient to induce locomotor bursting on the contralateral side of the spinal cord (Kjaerulff and Kiehn, 1997). Intracellular recordings from motoneurons on the non-locomoting side revealed rhythmic barrages of inhibitory postsynaptic potentials (IPSPs) which were blocked by application of strychnine, but not bicuculline, to the non-locomoting side, revealing the crossed inhibition of motoneurons during fictive locomotion to be glycinergic in nature (Kjaerulff and Kiehn, 1997). These IPSPs were also blocked by application of APV (NMDA receptor antagonist) or CNQX (AMPA receptor antagonist) to the non-locomoting side, illustrating that glutamatergic transmission is a requirement for the rhythmic inhibition of motoneuron (Kjaerulff and Kiehn, 1997). This means that despite the known presence of monosynaptic commissural inhibitory inputs to motoneurons, the crossed inhibition that is actually occurring during fictive locomotion is disynaptic (or polysynaptic) in nature, and at some point, after crossing the midline, glutamatergic transmission is required (Kjaerulff and Kiehn, 1997).

Part III: Molecular dissection of the spinal cord.

Although significant strides have been made regarding knowledge of the general location and pharmacology of the locomotor CPG, relatively little progress has been made identifying specific interneuronal components of this network, or the manner in which they are interconnected to form a functional circuit. Two major obstacles in this regard have been a) the large number of interneurons present in the mammalian spinal cord; and b) the inability to identify populations of interneurons with a given function. Recent advances in developmental neurobiology have provided novel tools to study the identity and function of specific interneuronal populations. These tools have provided key insights into the structure and mechanism of function of the locomotor CPG (Grossman *et al.* 2010).

Neuronal differentiation in the ventral horn of the mammalian neural tube is determined by the secretion of the morphogen Sonic Hedgehog (Shh) from the notochord and floor plate during embryogenesis. This results in the establishment of a Shh concentration gradient along the dorsal-ventral axis with neuronal progenitor cells located in the ventral-most regions of the spinal cord being exposed to significantly higher concentrations of Shh than those situated dorsally (Echelard *et al.* 1993; Krauss *et al.* 1993; Marti *et al.* 1995; Roelink *et al.* 1994, 1995; Tanabe *et al.* 1995; Ericson *et al.* 1995, 1997). Shh signalling is dependent on the binding of Shh ligands to transmembrane receptor proteins of the Patched family which, when bound to Shh, lose their ability to bind and repress the activity of a second transmembrane protein, Smoothened (Alcedo *et al.* 1996;

Chen and Struhl 1996; Marigo *et al.* 1996; Stone *et al.* 1996; van den Heuvel and Ingham 1996). Derepression of Smoothened triggers an intracellular signalling cascade that results in the activation of transcription factors including zinc finger transcription factors of the Gli/Ci family (Alexandre *et al.* 1996; Dominguez *et al.* 1996; Lee *et al.* 1997; Von Ohlen *et al.* 1997). The concentration of Shh that a neuronal progenitor cell is exposed to determines the specific host of transcription factors that are expressed in that cell. Transcription factors drive the expression of key genes that are responsible for the differentiation, migration pattern, and ultimately, function of a neuron (Ericson *et al.* 1997). The process of ventral neuron differentiation, triggered by Shh release from the notochord, is mirrored by the process of dorsal neuron differentiation, which is triggered by the release of bone morphogenic proteins (BMP's, specifically BMP-4 and BMP-7) by the dorsal ectoderm, which generates a concentration gradient with higher concentrations of BMP's in the dorsal regions of the developing spinal cord (Liem *et al.* 1997). Ultimately, eleven genetically-distinct populations of neurons have been identified in the spinal cord: the dorsal interneurons (dI1-dI6), the ventral interneurons (V0-V3), and the motoneurons (MN) (**Figure 5**) (for review see Kiehn 2010; Garcia-Campmany *et al.* 2010; Stepien and Arber 2008). Work on the roles of each of the ventral interneuronal populations V0-V3 in locomotor pattern formation and rhythm generation are outlined below.

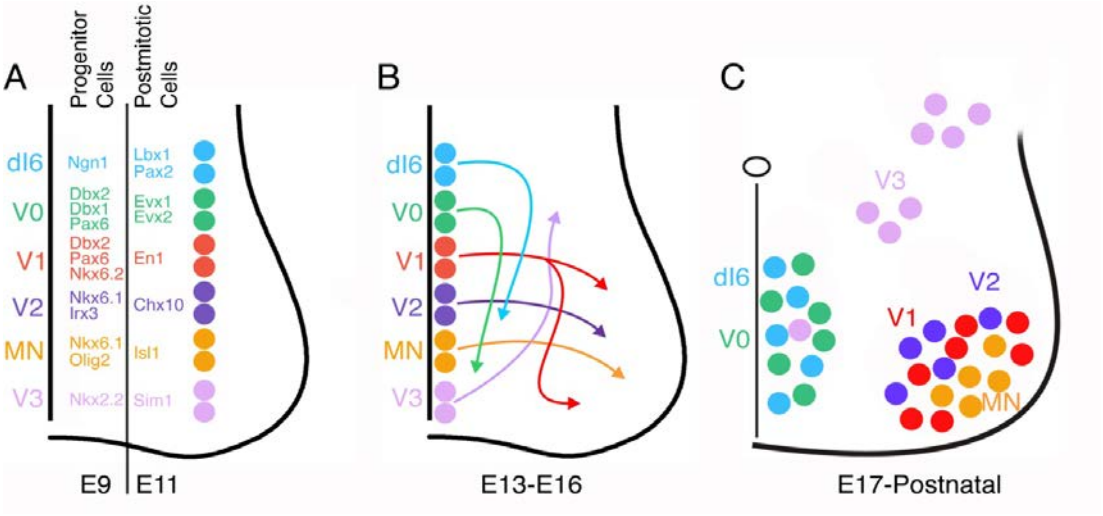

Figure 5: Neuronal populations of the ventral horn. Panel A: Early transcription factors (TFs) that define the fate of the five identified neuronal populations of the ventral horn (V0-V3 interneurons and the motor neurons, MN), as well as one dorsal interneuron population which migrates ventrally (dI6). To the left are listed TFs expressed by progenitor cells (ie. pre-mitosis), at embryonic day ~9 (E9). To the right are listed TFs expressed by postmitotic cells, at embryonic day ~11 (E11). **Panel B:** Progenitor positioning along the central canal and postmitotic migratory paths of the five neuronal populations of the ventral horn, and the dI6 dorsal interneurons. Migration occurs from embryonic day 13 through 16. **Panel C:** Final location of the five neuronal populations of the ventral horn, as well as the dI6 dorsal interneurons, following migration.

V0 Interneurons

The V0 interneuron population originates at the border of the dorsal and ventral neural tube, and migrates toward the ventromedial region of the spinal cord (lamina VIII). V0 progenitor cells are defined primarily by the expression of the transcription factor Dbx1, but they also express Dbx2 and Pax6 (Pierani *et al.* 1999; Pierani *et al.* 2001; Moran-Rivard *et al.* 2001). The axons of V0 interneurons project commissurally, crossing the midline before joining the ventrolateral funiculus (VLF) and extending either rostrally or caudally before reentering the grey matter (Pierani *et al.* 2001; Moran-Rivard *et al.* 2001). Postmitotic V0 cells can be further divided into three subpopulations based on transcription factor expression. $V0_V$ interneurons are derived from the ventral portion of the V0 progenitor domain and express the transcription factors Dbx1 and Evx1 (Burrill *et al.* 1997; Pierani *et al.* 1999; Pierani *et al.* 2001; Moran-Rivard *et al.* 2001; Lanuza *et al.* 2004). $V0_D$ interneurons are derived from the dorsal portion of the V0 progenitor domain and express Dbx1 but do not express Evx1 (Pierani *et al.* 1999; Pierani *et al.* 2001; Lanuza *et al.* 2004). The recently identified $V0_C$ interneurons are a very minor (~5% of V0 cells) subpopulation of interneurons localized immediately adjacent to the central canal that express Dbx1, Evx1 (thus they are a subset of the $V0_V$ population), as well as Pitx2 (Zagoraiou et al. 2009). Genetic knockout of the *Dbx1* gene results in the functional loss of all V0 interneurons ($V0_C$, $V0_V$ and $V0_D$), and the respecification of would-be V0 progenitor cells into neurons that are similar to the surrounding V1 and dI6 populations (Pierani *et al.* 2001; Lanuza *et al.* 2004). This loss of V0

interneurons does not affect the capability of the locomotor CPG to produce bursting activity in an *in vitro* locomotor preparation. Nor does it affect the alternation of ipsilateral flexor and extensor motoneurons. However, it does result in a complete loss of contralateral coordination, indicating that the V0 interneurons are an integral component of the locomotor CPG circuitry whose primary role is the coordination of left-right alternation (Lanuza *et al.* 2004). Genetic knockout of the *Evx1* gene alone results in the acute and specific loss of all $V0_V$ interneurons, leaving the $V0_D$ population intact. Unlike the *Dbx1* knockouts, the loss of Evx1-positive $V0_V$ neurons has no effect on fictive locomotor activity, suggesting that the loss of left-right alternation observed in the *Dbx1* knockout experiments can be attributed specifically to the loss of the $V0_D$ interneuron population (Lanuza *et al.* 2004).

V1 interneurons

The V1 interneuron population is defined by postmitotic expression of the transcription factor En1. V1 progenitors express the transcription factors Dbx2, Pax6, and Nkx6.2 (Ericson *et al.* 1997; Matise and Joyner 1997; Saueressig *et al.* 1999; Vallstedt *et al.* 2001; Sapir *et al.* 2004). V1 interneurons are inhibitory, migrate ventrolaterally to lamina VII of the postnatal spinal cord, and have a very stereotypical projection pattern in which they extend their axons ventrally into the ipsilateral VLF, then rostrally for 1-2 segments before terminating near motoneurons (Saueressig *et al.* 1999; Sapir *et al.* 2004; Li *et al.* 2004; Higashijima *et al.* 2004). A subset of V1 interneurons has been shown to make up the functionally well-defined population of interneurons known as Renshaw Cells

(Renshaw, 1946; Sapir *et al.* 2004), which receive strong excitatory input from motoneuron axon collaterals and in turn inhibit motoneurons as well as Ia inhibitory interneurons (Eccles *et al.* 1954; Hultborn *et al.* 1971; Cullheim and Kellerth 1982; Arvidsson *et al.* 1992; Schneider and Fyffe, 1992; Alvarez *et al.* 1999). Selective silencing, or ablation of the entire V1 population, results in a severe increase in burst duration in the *in vitro* fictive locomotor preparation, and adult *En1* knockout mice exhibit profound difficulty walking at high speeds (Gosgnach *et al.* 2006). These results suggest that V1 interneurons are a component of the locomotor CPG that are involved in determining the speed of locomotion (Gosgnach *et al.* 2004).

V2 interneurons

V2 interneuron progenitor cells express Lhx3 in the absence of Isl1 (Ericson *et al.* 1997; Zhou *et al.* 2000; Karunaratne *et al.* 2002; Smith *et al.* 2002; Peng *et al.* 2007) and migrate to a ventrolateral position, intermingled with the V1 cells. Postmitotic V2 interneurons can be classified into two subpopulations: the V2a interneurons, which are ipsilaterally-projecting, glutamatergic interneurons defined by the expression of the transcription factor Chx10, and the V2b interneurons, which are ipsilaterally-projecting inhibitory interneurons defined by the expression of the transcription factors Gata2 and Gata3 (Ericson *et al.* 1997; Li *et al.* 2005; Lundfald *et al.* 2007; Al-Mosawie *et al.* 2007). While the characteristics and function of the V2b cells have not investigated, ablation of the V2a subpopulation disrupts left-right alternation of locomotion *in vitro*, and increases the variability of burst amplitude and cycle period (Crone *et al.* 2008).

Adult mice normally use a "trotting" gait (left-right alternation) while running at any speed, but mice in which Chx10+ interneurons have been ablated tend to switch to a "galloping" gait (left-right synchrony) at higher speeds of treadmill locomotion (Crone *et al.* 2009). Whole-cell recordings from V2a neurons demonstrate that roughly 50-65% of V2a interneurons at the L2 level are rhythmically-active during fictive locomotion (Zhong *et al.* 2010; Dougherty *et al.* 2010). Anatomical analysis of this population has demonstrated that they provide excitatory input to commissural interneurons (including the V0 population). Thus, it has been postulated that V2a interneurons function in the control of left-right alternation of locomotion at high walking speeds by exerting control over commissural interneurons (Crone *et al.* 2008; Crone *et al.* 2009).

V3 interneurons

The V3 interneuron population is defined by the postmitotic expression of the transcription factor Sim1 (Briscoe *et al.* 1999; Goulding *et al.* 2002). These cells migrate dorsolaterally, are excitatory, and form synapses onto ventral interneurons and motoneurons. The majority of V3 interneurons are commissural, although ~15% project ipsilaterally and a small portion extend bifurcating axons that project both ipsilaterally and commissurally (Zhang *et al.* 2008). Selective silencing of the V3 population disrupts the regularity of the locomotor rhythm (Zhang *et al.* 2008). Additionally, locomotor bursting *in vitro* shows increased variability in the step cycle period, burst duration, and interburst period, and the left and right sides of the cord begin to produce assymetrical bursting activities (Zhang *et al.* 2008). Based on these data it was suggested that the V3 interneurons

play a role in the establishment of a robust and balanced locomotor rhythm (Zhang *et al.* 2008).

Although the aforementioned work incorporating molecular genetic techniques together with electrophysiology has provided a great deal of information about the component interneurons of the locomotor CPG and their specific roles during locomotion, several key functions have yet to be ascribed to specific interneuronal populations. These include the coordination of ipsilateral alternation of flexor and extensor motoneuron pools, and the initiation of rhythmic activity in the locomotor CPG. In this thesis I will attempt to identify neuronal populations responsible for the initiation of rhythmic activity in the locomotor CPG, specifically by examining whether a small subpopulation of V0 interneurons may be involved.

Thesis Work: Role of the V0 interneurons in the initiation of locomotion.

Introduction

The V0 interneurons are defined by the expression of the transcription factor Dbx1, migrate to the ventromedial region of the spinal cord settling in or near lamina VIII, and project commissurally (Pierani *et al*. 1999, 2001; Moran-Rivard *et al.* 2001). A substantial proportion of these cells make monosynaptic contacts onto contralateral motoneurons (Lanuza et al. 2004). They are also vital to the coordination of left-right alternation during fictive locomotion, a function that likely relies on the subset (~70%) of V0 interneurons which are inhibitory (Lanuza et al. 2004). In spite of the extensive work investigating this population, a function has not yet been assigned to the 30% of V0 interneurons that are excitatory.

The initiation of locomotion *in vivo* is dependent on input that descends to the locomotor CPG via the brainstem reticular formation. The critical nuclei are the nucleus reticularis gigantocellularis (NRGC) and the nucleus reticularis magnocellularis (NRMC) (Schefchyk *et al*. 1984; Orlovsky *et al.* 1999; Noga *et al.* 2003; Matsuyama *et al.* 2004). NRGC and NRMC neurons receive inputs from three higher brain nuclei which, when electrically stimulated, are sufficient to initiate locomotor rhythmogenesis: the subthalamic locomotor region (SLR) (Orlovsky, 1969); the fastigial nucleus of the cerebellum (Walberg *et al*. 1962); and the mesencephalic locomotor region (MLR) (Baev *et al*. 1988). Anatomical investigations in the cat have revealed that the neurons of NRGC and NRMC send

their axons to the spinal cord via one of three tracts in the ventrolateral funiculus (VLF): the ventromedial reticulospinal tract (RSTm), the ipsilateral lateral reticulospinal tract (RSTi), and the contralateral lateral reticulospinal tract (RSTc) (Peterson *et al*. 1975). These projections terminate in the ventromedial regions of the cervical and lumbar enlargements, forming synapses with neurons in, or near, lamina VIII (Peterson *et al.* 1975). Perhaps the strongest evidence for the role of the reticular nuclei as a relay centre between cortical command regions and the cells of the locomotor CPG is the demonstration that reversible cooling of either the reticular formation or the VLF - a procedure which blocks neurotransmission from passing through these structures - abolishes MLR-induced locomotor activity in vivo (Schefchyk *et al*. 1984; Noga *et al.* 2003).

Based on their location in lamina VIII of the lumbar spinal cord, I hypothesize that the excitatory V0 interneurons act as first-order interneurons of the locomotor CPG, and play a role in the initiation of rhythmic locomotor activity in the spinal cord. Here I use anterograde tracing of reticulospinal axons to demonstrate that these cells make monosynaptic contacts onto V0 interneurons. I have also used immunohistochemistry to show that V0 interneurons express both 5-HT_7 and 5-HT_{2A} receptor subtypes, both of which are required for the initiation of locomotion, as is illustrated by application of antagonists for either receptor subtype in an *in vitro* fictive locomotor preparation (Liu and Jordan, 2005). These results support my hypothesis that the V0 interneurons are first-order interneurons of the locomotor CPG that signal the initiation of rhythmic locomotor activity.

Methods:

Transgenic Mice

In order to visualize the V0 interneurons, a heterozygous *Dbx1*$^{LacZ/+}$ knock-in transgenic mouse line was utilized in which one copy of the *Dbx1* gene has been removed and replaced with a *LacZ* reporter gene (Pierani et al. 2001; Moran-Rivard *et al.* 2001). In knock-in transgenic lines such as this, the transgene (*LacZ*) locus is identical to the locus of the target gene (*Dbx1*), meaning the *LacZ* transgene is not only paired with the *Dbx1* promoter sequence, but is also paired with both nearby and distant regulatory gene sequences, and undergoes identical patterns of chromatin packaging regulation. This results in an expression pattern for the reporter gene that very closely matches the expression pattern of the target gene *Dbx1*.

Genotyping

Mice were genotyped using Polymerase Chain Reaction (PCR) technique. Tail biopsies were first subjected to a proteinase K (Fermentas) digestion, and DNA was precipitated by isopropanol, isolated, washed in 70% EtOh, and redissolved in H_2O. PCR was then performed on a DNA sample. Taq DNA polymerase and associated reagents were purchased from Roche; temperature cycling was performed with a BioRad MJ Mini personal thermal cycler (annealing temperature for *LacZ* is 64º C). *LacZ* forward primer sequence: TTGGCGTAAGTGAAGCGAC. *LacZ* reverse primer sequence: AGCGGCTGATGTTGAACTG.

Dye Application

Dbx1$^{LacZ/+}$ mice were taken at postnatal day 0-2 (older mice show weak β-Gal expression), anaesthetized using an isofluorane chamber, and dissected in oxygenated low-Ca^{2+} Ringers dissection solution (composed of 111 mM NaCl, 3.08 mM KCl, 11 mM glucose, 25 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 3.7 mM $MgSO_4$, and 0.25 mM $CaCl_2$). A laminectomy was performed in order to extract the intact brainstem and spinal cord. Crystals of the carbocyanine dye Fast DiI (1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate; Invitrogen) were placed in the reticular formation of the brainstem. FAST DiI is a bleach-resistant carbocyanine dye that is an effective retrograde and anterograde axonal tracer; it is lipophilic and is taken up by cells via insertion into the outer leaflet of the cell membrane, and is transported both by active vesicular lipid turnover (in living tissue) and by passive diffusion along the membrane, allowing for complete extension of cellular processes and temperature-dependant transport in fixed tissue (Köbbert et al. 2000). Initial measurements based on anatomical atlas study and light microscopy of Nissl-stained slides estimated optimum crystal placement site (center of the nucleus reticularis gigantocellularis) to be 0.4-0.8 mm from midline, 0.4 mm caudal to the caudal margin of the cerebellum, and 1.1-1.4 mm deep (**Figure 6, lower panels**). More precise dye placement techniques using stereotaxic apparati are only feasible in adult mice and thus were not used.

Dye Transport

In initial trials, the dye transport method of Sparks *et al*. (2000) was used.

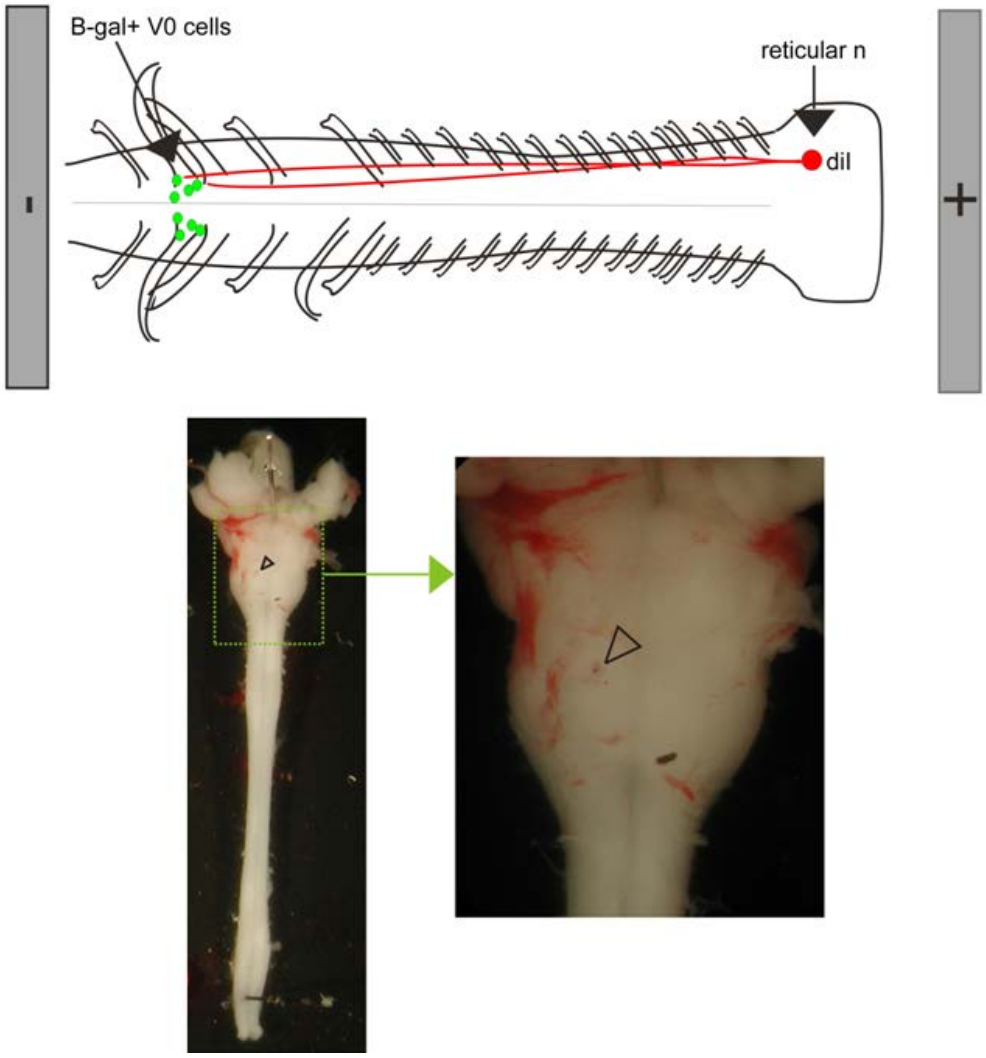

Figure 6: DiI implant site and parallel plate capacitor used to facilitate DiI transport. Lower Panels: Photographs showing the DiI crystal implantation site. Crystals of DiI were implanted into the brainstem reticular formation at the site of the Nucleus Reticularis Gigantocellularis, located via the Allen neonatal mouse brain atlas. Coordinates of the DiI implant are: 0.4-0.8 mm from midline, 0.4 mm caudal to the caudal margin of the cerebellum, and 1.1-1.4 mm deep. Uptake of the dye was facilitated by active transport while the tissue was kept alive in oxygenated Ringers solution for 24 hours. **Upper Panels:** Diagram showing the parallel plate capacitor setup used to accelerate DiI transport. Following PFA fixation, brainstem-spinal cord preparations were placed in a mineral oil bath, which acted as a dielectric in this parallel plate capacitor setup.

Incubation times were estimated from literature values and confirmed in initial transport-assay trials. DiI is a bidirectional neuronal tracer that is lipid-soluble and travels via diffusion in the phospholipid bilayer, is not transferred to adjacent neurons, and is capable of diffusing long distances (cm values) in fixed tissue (Sparks *et al*. 2000). DiI also undergoes active transport via vesicular lipid turnover in living tissue; therefore the tissue was kept alive for ~24 hours in oxygenated standard Ringers solution (composed of 111 mM NaCl, 3.08 mM KCl, 11 mM glucose, 25 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 1.25 mM $MgSO_4$, 2.52 mM $CaCl_2$) at 4 °C in order to allow active dye uptake and penetration. In initial trials, cords were then fixed and incubated in paraformaldehyde (PFA) for two to three weeks at 37 °C to allow for DiI to travel along reticulospinal axons the ~1.5 cm to the lumbar spinal cord. This prolonged incubation period is required under normal conditions, as the average rate of DiI diffusion in fixed tissue is ~0.012 mm/h (Swift *et al.* 2004; Lukas *et al.* 1998).

Initial trials revealed that after this prolonged incubation in fixative, the antigenicity of the *LacZ* gene product (the β-galactosidase enzyme) was interrupted, likely due to extensive crosslinking of proteins (Holmqvist *et al.* 1992), such that immunohistochemical staining techniques (see below) failed to allow these cells to be visualized. Such problems with antibody staining following prolonged fixative exposure have been noted before (Holmqvist *et al*. 1992). In seeking an alternative method it was found that despite being lipophilic, DiI carries a net positive charge and is thus responsive to an applied electrical field (Swift *et al.* 2004), increasing the speed of transport dramatically.

In a refined technique, the initial 24h incubation (see above) was followed with a short 1 hour fixation in PFA. The cords were then placed in a parallel-plate capacitor (2.5 cm distance between plates) using a mineral oil bath which acts as a dielectric insulator and also keeps the cords moist. The rostral end was oriented near to the positive plate. Cords were bathed in mineral oil (a dielectric insulator) for 48 hours at room temperature, under 40 V of potential (generating an electric field of approx. 16 V/cm) (**Figure 6, upper panels**). In order to ensure specificity of tract tracing, all of the spinal cord white matter except for the ventrolateral funiculus (VLF) was transected at the cervical or thoracic level **(Figure 7)**; this means that any aberrantly-labelled fibres would not be capable of transporting DiI caudally past the point of transection, and thus only VLF axons originating in the reticular formation would be observed in the lumbar spinal cord tissue. Under this applied electric field, the expected rate of DiI diffusion is ~0.50 mm/h; at this rate, it was estimated that it would take ~ 40h for the DiI to travel a distance of 2 cm, the approximate length of the brainstem-spinal cord preparations at P1 (Swift *et al.* 2004). Immediately following dye transport, brainstem-spinal cord preparations were washed with phosphate-buffered saline (PBS) 5 times for 10 minutes to remove mineral oil. It should be noted that if mineral oil was not sufficiently washed away, problems occurred during cryostat sectioning, as the mineral oil interrupted the bond between the tissue and the OCT compound.

Immunohistochemistry

PFA-fixed tissue was cryoprotected in 20% sucrose at 4°C 4-8 hours and flash-frozen in Optimal Cutting Temperature (OCT) compound (Tissue-Tek). 20

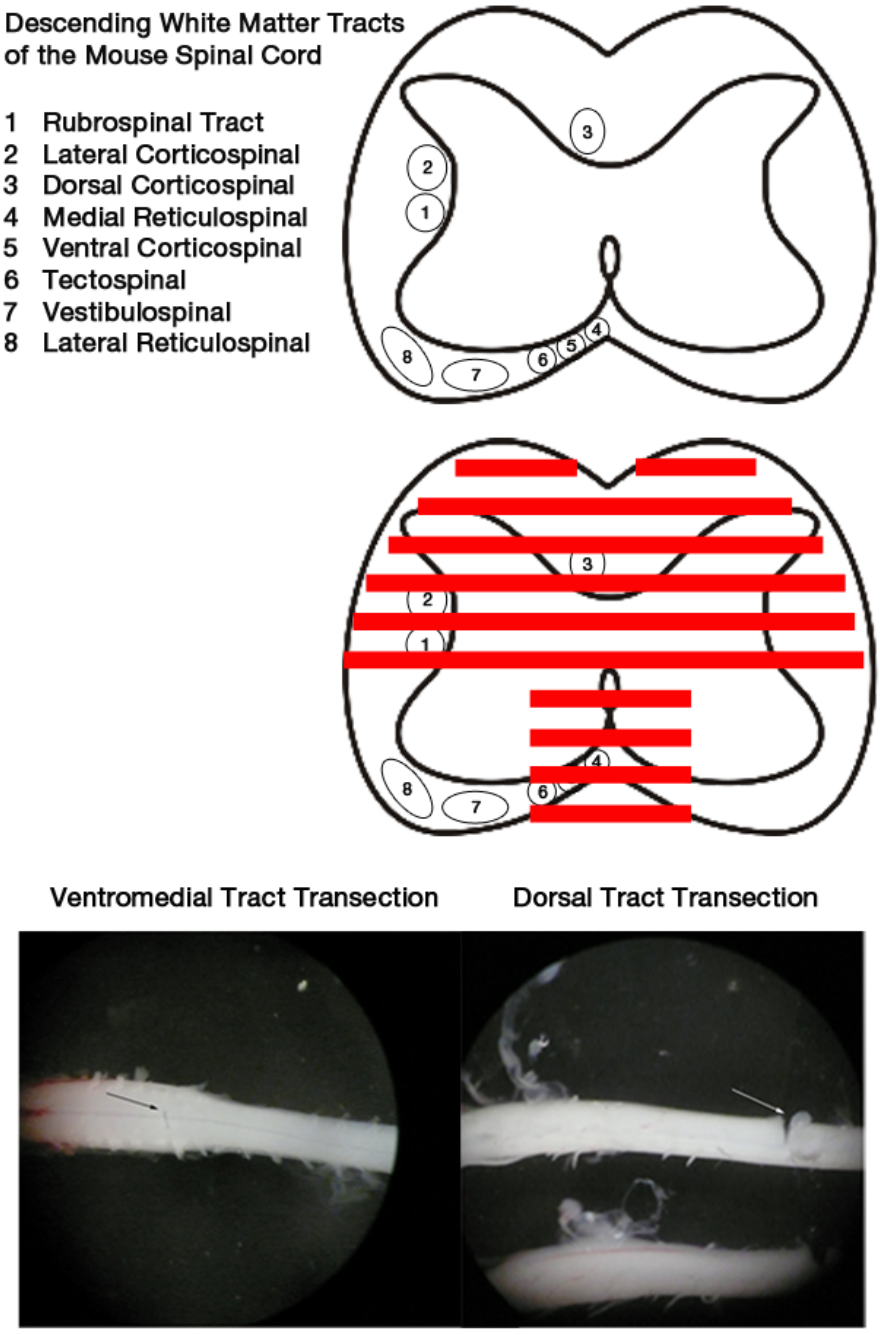

Figure 7: Diagram of tract transections performed prior to DiI transport. Prior to DiI transport, all of the white matter tracts that do not lie within the ventrolateral funiculus were transected at the cervical or thoracic level. **Upper Panel:** Diagram showing the major descending white matter tracts of the mouse spinal cord. **Middle Panel:** Diagram depicting the regions of white matter to be transected (crossed out in red). **Lower Panels:** Dissecting microscope photographs of the preparation following tract transections.

μm sections were cut on a cryostat at -20°C. Immunohistochemistry was performed using the following primary antibodies and dilutions: rat (1:1000) and mouse (1:50) anti-β-gal (gifts from the Goulding lab – Salk Institute for Biological Studies); rabbit anti-synaptotagmin (1:500) (Alomone Labs); rabbit anti-$5HT_7$ receptor (1:500) (AbCam); rabbit anti-$5HT_{2A}$ receptor (1:500) (AbCam); and sheep anti-c-Fos (1:100) (Millipore). Secondary antibodies used were as follows: goat anti-rat-IgG (cy2-conjugated) (1:300) (AbCam); goat anti-rat-IgG (cy3-conjugated) (1:300) (AbCam); goat anti-rabbit-IgG (cy2-conjugated) (1:300) (AbCam); goat anti-rabbit-IgG (cy5-conjugated) (1:300) (AbCam); donkey anti-sheep-IgG (cy3-conjugated) (1:100) (Millipore); donkey anti-sheep-IgG (cy5-conjugated) (1:100) (Millipore); and donkey anti-rabbit-IgG (cy2-conjugated) (1:300) (Jackson Immunoresearch). Immunohistochemistry was performed via methods previously described (Gross *et al.* 2000; Lanuza *et al.* 2004). Images were captured using a Zeiss LSM 510 NLO laser scanning confocal microscope, and a Leica DM5500B fluorescence microscope. Images were analyzed and edited (brightness and contrast adjustments) using the following software: Zeiss LSM Image Browser (Zeiss), ImageJ (National Institutes of Health), and Adobe PhotoShop CS4 (Adobe).

Electrophysiology

P0–P3 wild-type (*CD1*; Charles River) or *Dbx1*$^{LacZ/+}$ mice were anaesthetized with isofluorane, euthanized by decapitation, and eviscerated. Spinal cords were dissected out via a ventral laminectomy in oxygenated ice-cold low-Ca^{2+} Ringer's solution (see above) and pinned ventral-side up in a recording chamber which was

perfused with oxygenated, room temperature, standard Ringer's solution (see above). Fictive locomotor activity was induced by bath application of NMDA (5µM) and 5-HT (10µM), and recorded via suction electrodes placed on the second (i.e. L2) and fifth (i.e. L5) lumbar ventral roots. Electroneurogram (ENG) activity recorded from suction electrode application to the ventral roots was amplified, filtered, digitized, and recorded on a PC running Axoscope software (Molecular Devices). After 1 hour of stable fictive locomotor activity, tissue was prepared for immunohistochemistry (see above). Post hoc labelling with an antibody for the product of the immediate early gene c-*fos*, which is specifically expressed by neurons in response to the firing of action potentials, allowed for the identification of those cells that were active during fictive locomotion (Kiehn and Kjaerulff, 1998; Armstrong and Muntmini, 1993; Lanuza *et al.* 2004; Munglani and Hunt, 1995; Dai *et al.* 2005). Control experiments were performed in which addition of NMDA and 5-HT was omitted to ensure that c-Fos was an effective and accurate marker of cells that were active during fictive locomotion.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences between means were compared using raw data and either a one-way ANOVA F-test with Tukey-Kramer multiple comparison *post hoc* analysis, or a dependent *t*-test for paired samples (Devore and Peck, 2001).

Results

The reticular formation makes monosynaptic contacts onto V0 interneurons.

The reticular formation is the final relay point onto which various locomotor-initiating sites in the brain/brainstem synapse before descending to the locomotor CPG (Schefchyk et al. 1984). In order to determine whether reticular formation neurons make direct contacts onto V0 cells in the lumbar spinal cord, the anterograde tracer DiI was applied to this nucleus and, following dye transport, the lumbar spinal cord was inspected to determine whether labeled synapses were located in close proximity to V0 interneurons. V0 cells could be easily identified in the *Dbx1*$^{LacZ/+}$ mouse line in which β-gal is selectively expressed in all V0 interneurons (Pierani et al. 2001; Lanuza et al. 2004). Initial experiments indicated that DiI reached the lumbar spinal cord 48h after application of the DiI crystal, and the preparation was typically left in the parallel plate capacitor for 72h, to maximize the number of reticular neurons that had taken up the tracer. At this point, sectioning the cervical cord in the coronal and sagittal planes indicated that the DiI-labelled axons were clearly visible in the ventrolateral funiculus, the tract in which reticular formation neurons descend through the spinal cord **(Figure 8)**. Furthermore, individual, defasciculated axons could be observed in the grey matter of low thoracic spinal cord tissue **(Figure 9)**. These observations indicate that DiI transport procedures were successful in labeling neurons of the reticular formation from their point of origin down to the caudal spinal cord.

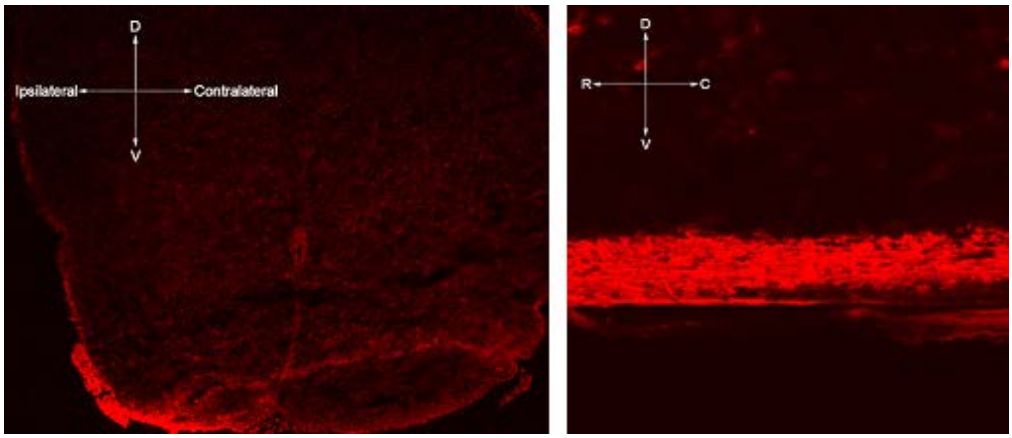

Figure 8: DiI labelling of the ventrolateral funiculus in cervical sections. Following DiI application and transport, the ventrolateral funiculus (VLF) was brightly labelled in cervical sections. **Left Panel:** Photomicrograph (10x magnification) of a coronal section taken from cervical spinal cord tissue following DiI transport. Ipsilateral to the site of DiI application, the VLF is brightly labelled. Contralateral to the site of DiI application, no labelling is seen. **Right Panel:** Photomicrograph (20x magnification) of a sagittal section taken from cervical spinal cord tissue following DiI transport, ipsilateral to the site of DiI application. The VLF is brightly labelled.

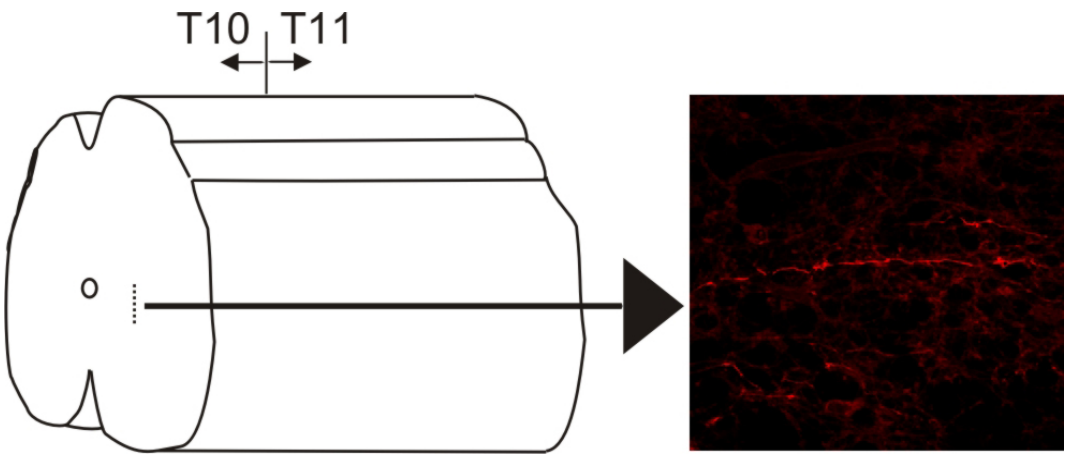

Figure 9: Individual axons labelled in thoracic grey matter. Photomicrograph (right panel, 40x magnification) of a horizontal section taken from T10 thoracic spinal cord tissue following DiI application and transport. Photomicrograph shows the ventromedial region of the lower thoracic spinal cord (approximate location indicated by dashed line in left panel). Ipsilateral to the site of DiI application, an individual axon can be observed travelling through the grey matter. This axon has defasciculated, leaving the VLF and entering the grey matter in order to approach its target cell.

In coronal sections taken from the lumbar spinal cord of *Dbx1*$^{LacZ/+}$ mice, antibody stains for β-Gal and synaptotagmin, a molecule that is specifically expressed in presynaptic axon terminals, indicated that individual DiI-labelled axons terminate in close proximity to V0 cell nuclei in the lumbar spinal cord **(Figure 10, Figure 11)**. Axons which terminated within 2 μm of a V0 cell nucleus were considered to form synapses onto their respective V0 interneuronal targets. Synaptotagmin was used to ensure that terminals were labeled, rather than simply axons of passage**.** In many cases, Z-Stack reconstructions revealed DiI^{+} axons as they approach a V0 cell nucleus from a distance of up to 1 μm, with synaptotagmin labeling appearing only as the axon comes into close proximity to the V0 cell, providing strong evidence that synapses were made onto these cells. It should be noted that in many cases, DiI^{+}, synaptotagmin^{+} axon terminals were observed in close apposition to cells other than V0 interneurons **(Figure 12)**, indicating that the V0 cells are not the sole target of reticulospinal projections. Overall, 32% (69/218) of the observed DiI^{+}, synaptotagmin^{+} terminals were found to be in close apposition to V0 cell nuclei or observed dendrites, with the remaining 68% (149/218) terminating close to a non-V0 cell. This ratio was conserved across spinal segments (T10-L5) and the total number of synapses observed was roughly equal on the sides of the spinal cord ipsilateral (111) and contralateral (107) to the site of tracer application. Overall, a greater number of DiI^{+}, synaptotagmin^{+} synaptic terminals were observed in more rostral tissue at T10-T12 (118), with fewer synaptic terminals appearing in more caudal tissue at L1-L3 (59) and L4-L6 (41).

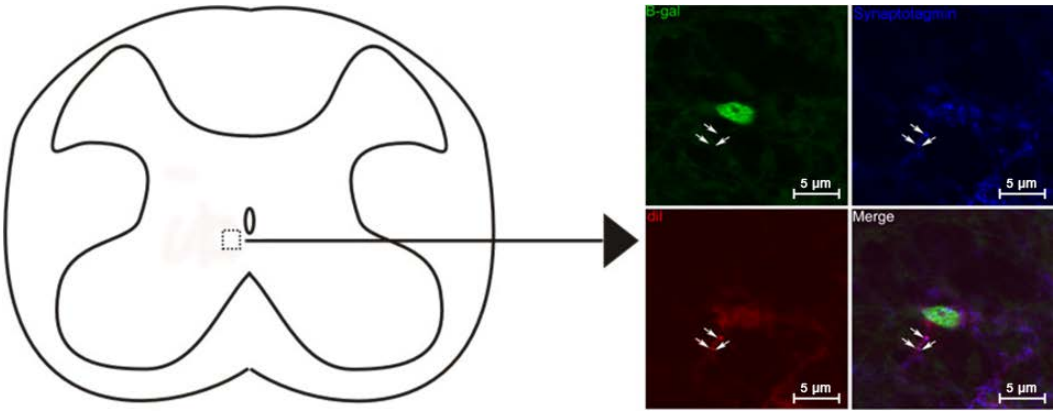

Figure 10: DiI-labeled axon terminals in close proximity to V0 cell nuclei. Photomicrograph (right panel, 40x magnification) taken from a coronal section of lamina VIII of the upper lumbar spinal cord (see dashed box in left panel). The nucleus of a β-Gal$^+$ V0 cell is labeled with a β-gal antibody (green). Synaptotagmin antibody labels synapses presynaptically (blue). DiI$^+$ projections (red) can be seen terminating in close apposition (within 2 µm) to the nucleus of the V0 cells as indicated by co-labeling of DiI$^+$ axons and synaptotagmin (white arrows) in merged panel.

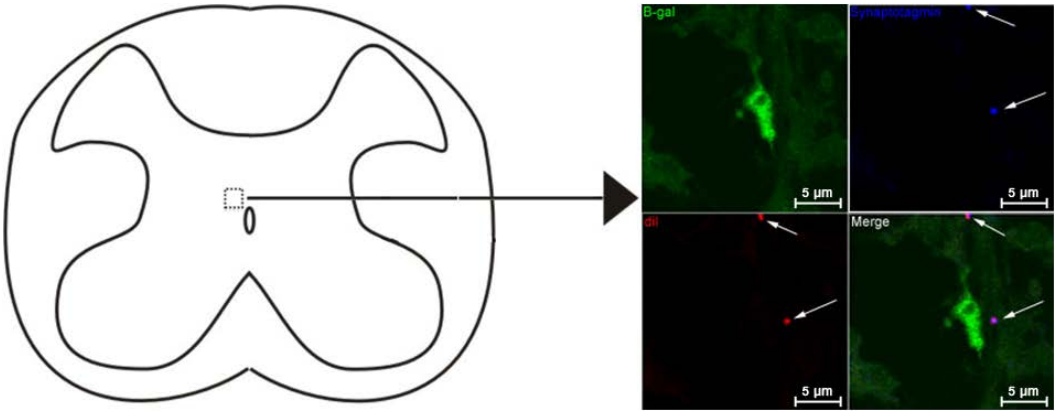

Figure 11: DiI-labeled axon terminals in close proximity to V0 cell nuclei. Photomicrograph (right panel, 40x magnification) taken from a coronal section of lamina X of the upper lumbar spinal cord (see dashed box in left panel). The nucleus of a β-Gal$^+$ V0 cell is labeled with a β-gal antibody (green). Synaptotagmin antibody labels synapses presynaptically (blue). DiI$^+$ projections (red) can be seen in close apposition (within 2 µm) to the nucleus of the V0 cell as indicated by co-labeling of DiI$^+$ axons and synaptotagmin (white arrows) in merged panel.

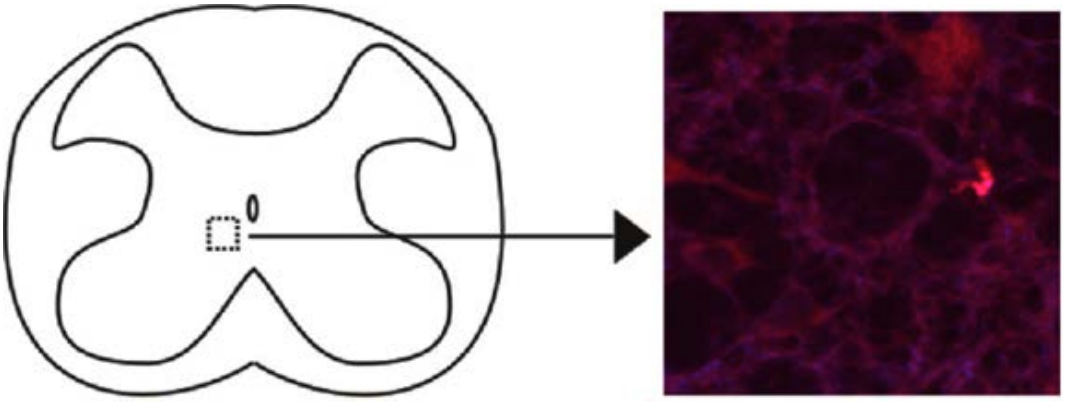

Figure 12: A DiI-labeled axon terminal in the absence of a V0 cell. Photomicrograph (right panel, 40x magnification) taken from a coronal section of lamina VIII of the upper lumbar spinal cord following DiI transport caudally from the reticular formation (see dashed box in left panel). Synaptotagmin antibody labels synapses (blue). DiI^{+} projections (red) can be seen colabelling with synaptotagmin, terminating in the absence of a green V0 cell.

The pooled data are illustrated in **Figure 13** in which a plot has been constructed showing the location of each V0 interneuron that was observed to be in close apposition to a DiI^{+}, $synaptotagmin^{+}$ axon terminal (i.e. a putative synapse) at different levels of the spinal cord**.**

V0 cells express 5-HT_{2A} and 5-HT_{7} receptors.

As mentioned above, serotonin (5-HT) plays a crucial role in activating spinal locomotor CPGs. Evidence supporting this comes from experiments showing that 5-HT is sufficient for activating locomotion in the isolated spinal cord *in vitro* (Cowley and Schmidt, 1994; 1997), as well as studies that have shown that activation of specific subtypes of 5-HT receptors (5-HT_{2A}, 5-HT_{7} receptors) is critical to the generation of locomotion and that application of a 5-HT_{7} receptor antagonist rostrally, or a 5-HT_{2A} receptor antagonist caudally, abolishes fictive locomotion (Liu and Jordan, 2005). If the V0 cells are involved in the initiation of rhythmic activity within the spinal cord it is expected that they possess these specific 5-HT receptor subtypes. To this end, antibody stains were performed for β-Gal along with 5-HT_{7} receptor or 5-HT_{2A} receptor in *Dbx1*$^{LacZ/+}$ transgenic mice.

Overall, a majority of V0 cells (64.6% ± 2.1%) co-labeled for the 5-HT_{7} receptor. Cell counts were performed in order to assess any differences in the expression of 5-HT_{7} receptors at different spinal cord levels (T11-L6) **(Figure 14)**. Interestingly, the proportion of V0 cells that coexpressed 5-HT_{7} receptors appeared to decrease in a rostral-caudal gradient from lower thoracic (T11,T12) to

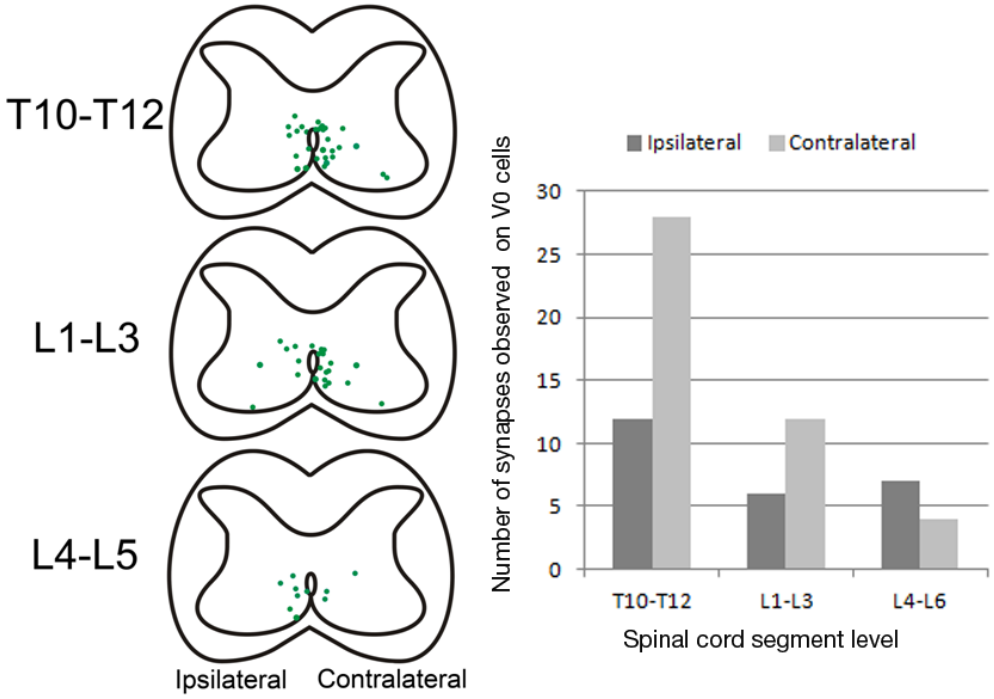

Figure 13: Schematic showing the distribution of observed synapses on V0 cells. Left Panel: In the above schematic, each green dot indicates the location of a V0 cell nucleus which was observed to be in close proximity to a DiI-labeled, synaptotagmin-labeled axon terminal, (i.e. observed putative 'synapses'). The diagram also shows whether the observed synapses were ipsilateral or contralateral to the site of DiI applications. Distribution of putative 'synapses' is shown for sections taken from lower thoracic (top panel), upper lumbar (middle panel) and lower lumbar (bottom panel) spinal cord. **Right panel:** Histogram showing the occurrence of observations of DiI-labeled, synaptotagmin-labeled axon terminals synapsing onto V0 cell nuclei at the different spinal cord segment levels. Bar colour indicates whether the observed synapses were ipsilateral or contralateral to the site of DiI application.

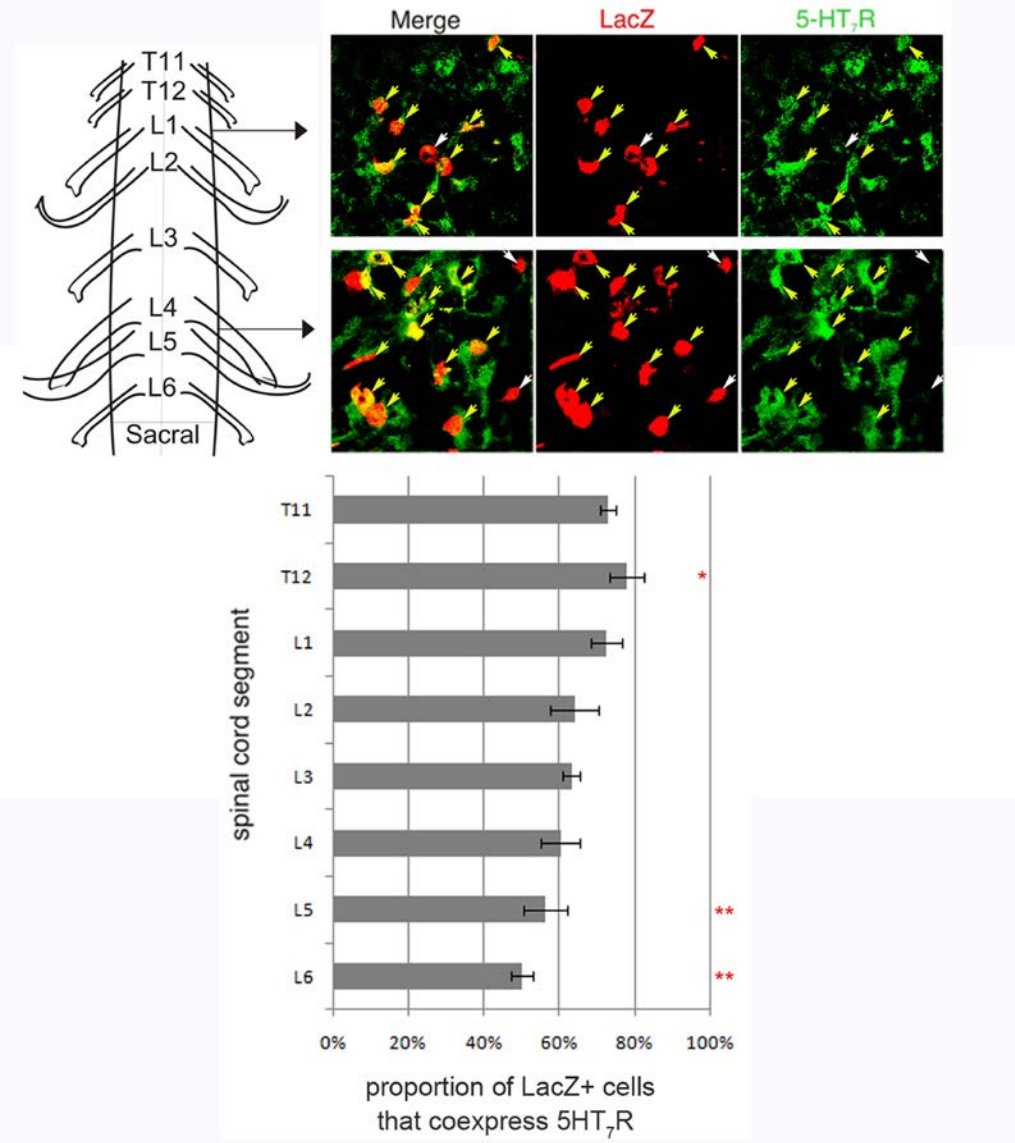

Figure 14: 5-HT$_7$ receptor staining colocalizes with V0 cell staining. Photomicrographs (right panels, 40x magnification) taken from lamina VIII of neonatal *Dbx1*$^{LacZ/+}$ mouse spinal cord (coronal sections) stained with antibodies to β-gal (red, labels V0 cell nuclei) and 5-HT$_7$ receptors (green). Note the presence of 5-HT$_7$ receptors (green punctae) on, and surrounding the V0 cell nuclei. V0 cells that colabel for 5-HT$_7$ receptors are indicated by yellow arrows. V0 cells that lack 5-HT$_7$ receptors are indicated by white arrows. Photomicrographs shown were taken at L1 (upper panels) and L4 (lower panels) segments of lumbar spinal cord. Graph (lower panel) represents mean proportion of β-Gal$^+$ cells that coexpress 5-HT$_7$ receptors at each spinal segment. Error bars represent standard error of the mean. Segments marked with one asterisk have significantly higher 5-HT$_7$ receptor levels than those segments marked with two asterisks ($0.01<P<0.05$).

lower lumbar (L5,L6) segments. In order to assess the significance of the observed rostral-caudal gradient, a one-way ANOVA F-test was performed; the F-test is designed to test the null hypothesis that a series of collected groups of data with different sample means actually have the same true mean, and thus could be considered one group. In this case, the null hypothesis was disproven by the F-Test ($F=3.12$, $F_{crit5\%}=2.34$, $0.01<P<0.05$), meaning that the expression of 5-HT_7 receptors by V0 interneurons differs significantly at different segmental levels. In order to specifically determine which sample groups differed significantly, the Tukey-Kramer multiple comparison procedure was performed. This analysis revealed significant differences in the number of V0 interneurons expressing 5-HT_7 receptors between the T12 and L5 segments, as well as between the T12 and L6 segments.

Antibody staining for the 5-HT_{2A} receptor revealed that the majority of β-Gal^+ V0 cells (68.0% ± 1.5%) expressed this receptor subtype. Cell counts were again performed to probe for differences in the proportion of V0 cells expressing the 5-HT_{2A} receptor at different spinal levels **(Figure 15).** In this case however, analysis via one-way ANOVA failed to disprove the null hypothesis, indicating that there are no significant differences in the proportion of V0 cells that expressed 5-HT_{2A} receptors at different segmental levels ($F=1.79$, $F_{crit5\%}=2.19$, $P>0.10$).

V0 cells that are active during locomotion express 5-HT_7 and 5-HT_{2A} receptors.

In order to focus my study on those V0 cells that were active during

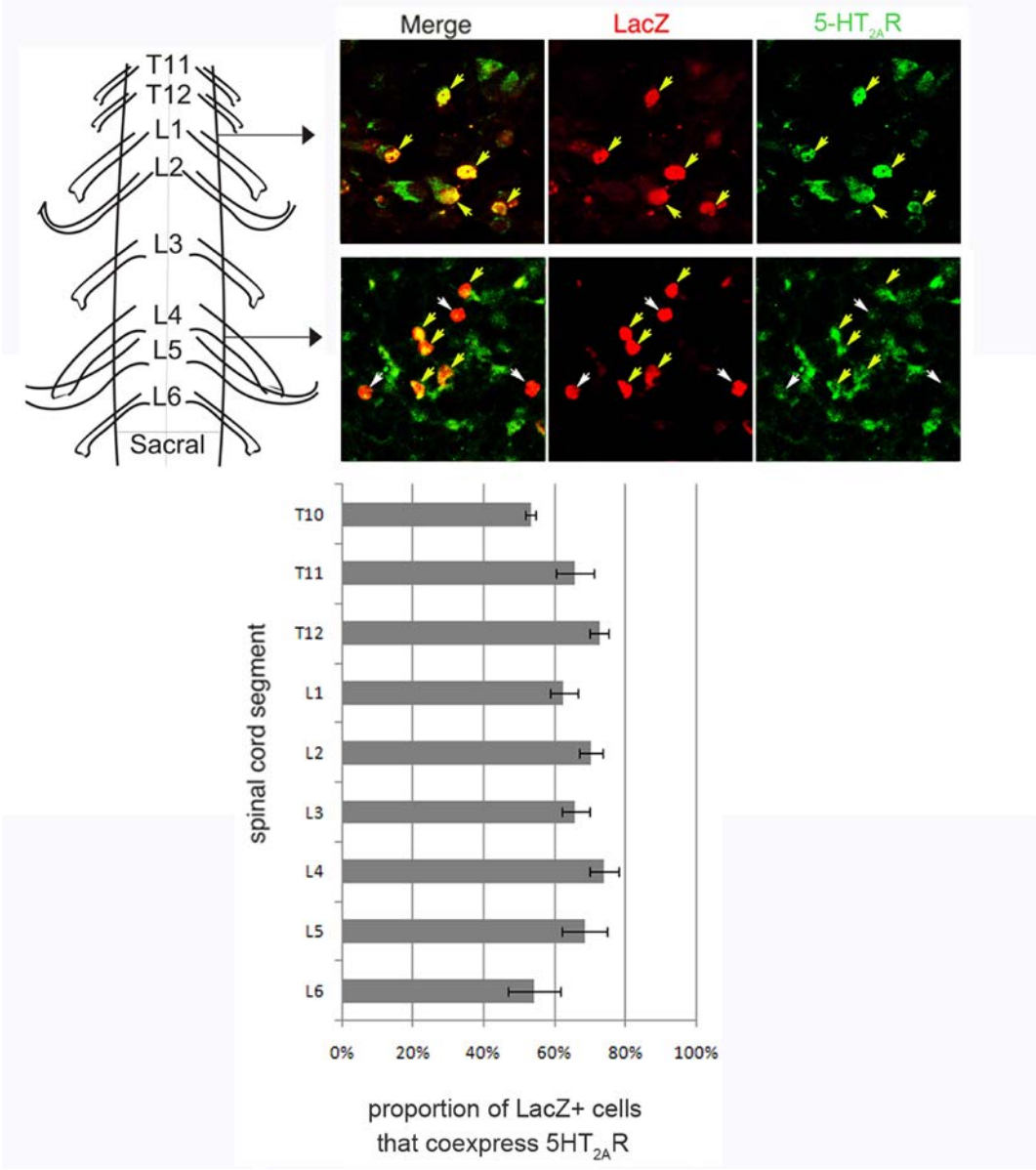

Figure 15: 5-HT$_{2A}$ receptor staining colocalizes with V0 cell staining. Photomicrographs (middle panels, 40x magnification) taken from lamina VIII of neonatal *Dbx1*$^{LacZ/+}$ mouse spinal cord (coronal sections) stained with antibodies to β-gal (red, labels V0 cell nuclei) and 5-HT$_{2A}$ receptors (green). Note the presence of 5-HT$_{2A}$ receptors (green punctae) on, and surrounding the V0 cell nuclei. V0 cells that colabel for 5-HT$_{2A}$ receptors are indicated by yellow arrows. V0 cells that lack 5-HT$_{2A}$ receptors are indicated by white arrows. Photomicrographs shown were taken at L1 (upper panels) and L3 (lower panels) segments of lumbar spinal cord. Graph (right panel) represents mean proportion of β-Gal^{+} cells that coexpress 5-HT$_{2A}$ receptors at each spinal segment. Error bars represent standard error of the mean.

locomotor activity, and thus potential components of the locomotor CPG, fictive locomotion was pharmacologically-induced via application of 5-HT and NMDA to the isolated spinal cord of neonatal *Dbx*$^{LacZ/+}$ mice. After 1 hour of rhythmic locomotor activity was recorded from the ventral roots (**Figure 16**), the spinal cords were quickly fixed and prepared for immunohistochemistry. Antibody staining for c-Fos has historically been effective at identifying cells that are active during locomotor activity (Lanuza et al. 2004), and combining this with antibodies for β-gal as well as 5-HT$_7$/5-HT$_{2A}$ receptors allowed for the identification of all V0 cells that are both active during locomotion, and express these receptors.

Following cell counts it was found that 56.6% ± 1.8% of V0 cells expressed both c-Fos and 5-HT$_7$ receptors (indicated by yellow arrows in **Figure 17**), 20.7% ± 1.3% of V0 cells expressed c-Fos but lacked 5-HT$_7$ receptors, 3.5% ± 0.6% of V0 cells lacked c-Fos expression but expressed 5-HT$_7$ receptors, and 19.2% ± 2.1% of V0 cells lacked both c-Fos and 5-HT$_7$ receptors (indicated by white arrows in **Figure 17**). These data appear to indicate that the population of V0 interneurons that are active during locomotion are more likely to express 5-HT$_7$ receptors than to lack them. In order to test the significance of this observation we performed a dependent *t*-test for paired samples. This test is used to determine whether there is a significant difference between the means of two separate measures taken from the same sample group, in this case the two measures being compared were the proportion of c-Fos-expressing V0 cells that coexpress 5-HT$_7$ receptors, and the proportion of c-Fos-expressing V0 cells that

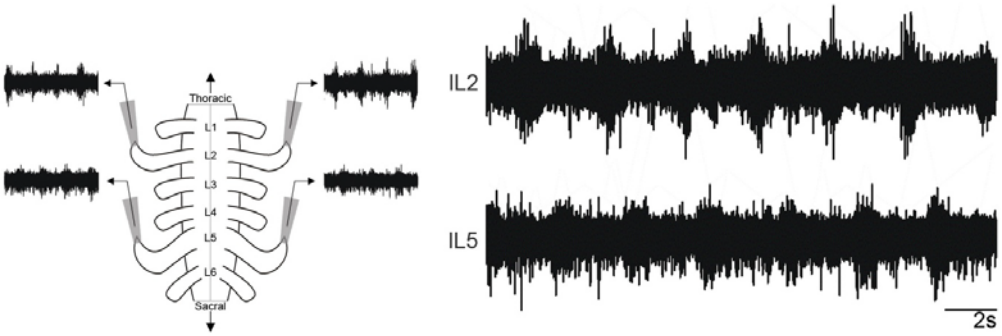

Figure 16: Ventral root recordings taken prior to c-Fos immunohistochemistry: In order to induce c-Fos expression in the spinal cord lfictive locomotion was induced in the isolated spinal cord by the addition of NMDA (5µM) and 5-HT (10µM). **Left Panel:** Diagram of the recording setup. **Right Panel:** Example of ventral root recordings evoked. Locomotor activity is characterized by rhythmic alternation between ipsilateral L2 roots (lL2), which consists mainly of flexor motor neuron axons, and ipsilateral L5 roots (lL5), which consist mainly of extensor motor neuron axons.

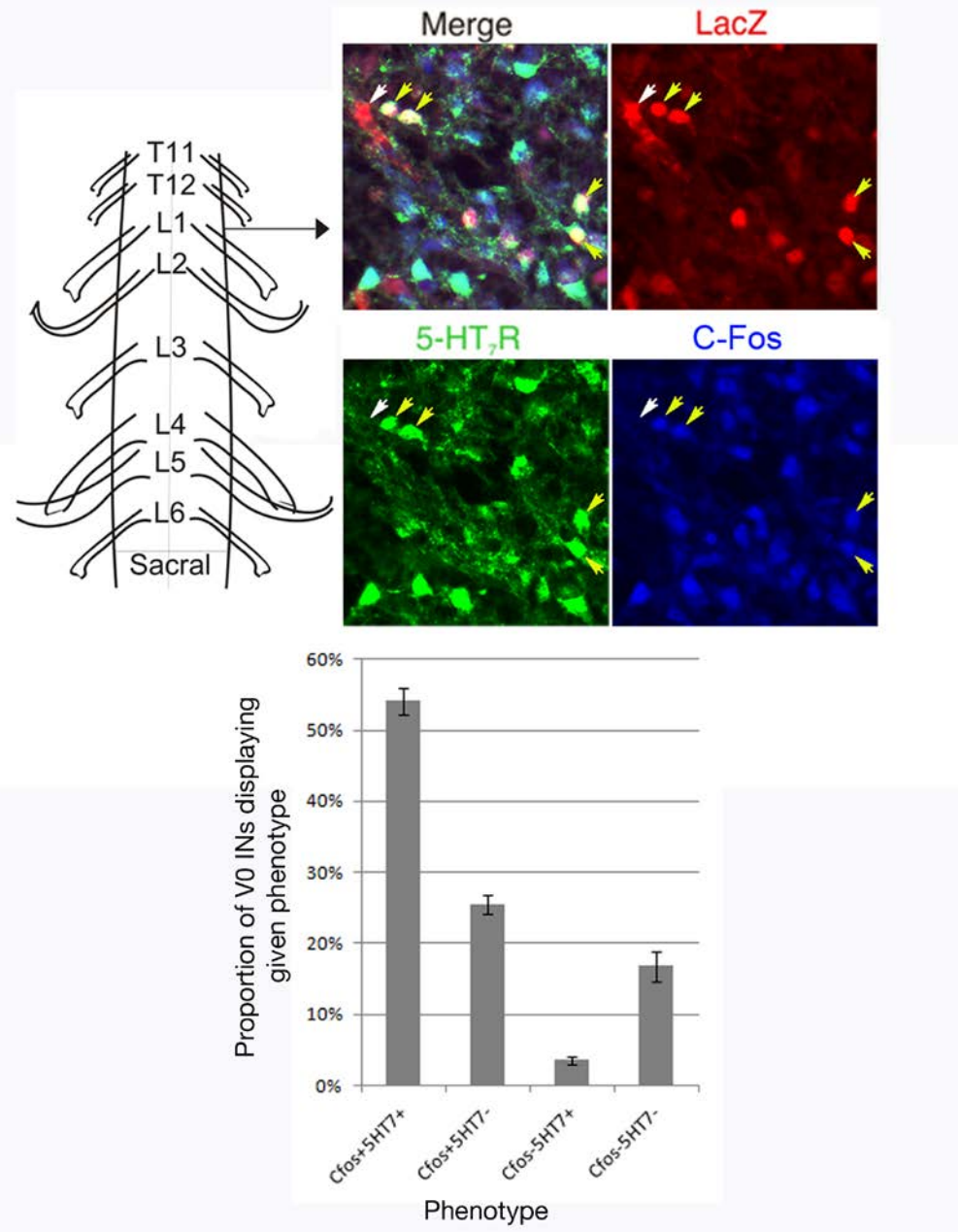

Figure 17: V0 cells that are active during locomotion express 5-HT$_7$ receptors. Top Panels: Photomicrographs (40x magnification) taken from lamina VIII of the neonatal *Dbx1*$^{LacZ/+}$ mouse lumbar spinal cord (coronal sections) that has undergone 1 hour of fictive locomotion, stained with antibodies for β-Gal (Red), 5-HT$_7$ receptor (Green), and c-Fos (Blue). V0 cells that are active during locomotion (ie. colabel for c-Fos) and also colabel for 5-HT$_7$ receptors are indicated by yellow arrows. V0 cells that lack c-Fos labelling and also lack 5-HT$_7$ receptors are indicated by white arrows. **Bottom Panel:** Histogram showing the relative proportions of V0 INs displaying each of the four possible phenotypes.

lack 5-HT_7 receptors. In this case the null hypothesis was disproven, supporting our observation that V0 cells that are active during locomotion are more likely to express 5-HT_7 receptors than to lack them (t=16.21, $t_{crit5\%}$=2.15, P<0.001). The data also revealed that the population of V0 interneurons that are not active during locomotion are more likely to lack 5-HT_7 receptors than to express them (t=5.80, $t_{crit5\%}$=2.15, P<0.001).

Repeating the same staining protocol probing for the 5-HT_{2A} (rather than 5-HT_7 receptors), allowed me to determine that 54.1% ± 2.6% of V0 cells express both c-Fos and 5-HT_{2A} receptors (yellow arrows in **Figure 18**), 25.6% ± 2.1% of V0 cells express c-Fos but not 5-HT_{2A} receptors, 3.6% ± 0.9% of V0 cells were found to lack c-Fos expression but possess 5-HT_{2A} receptors, and 16.8% ± 2.6% of V0 cells lacked both c-Fos and 5-HT_{2A} expression (white arrows in **Figure 18**). Thus the population of V0 interneurons that are active during locomotion tend to be more likely to express 5-HT_{2A} receptors than not (t=7.56, $t_{crit5\%}$=2.15, p<0.001) while V0 cells that were inactive during locomotion were more likely to lack 5-HT_{2A} receptors than to express them (t=5.08, $t_{crit5\%}$=2.15, p<0.001).

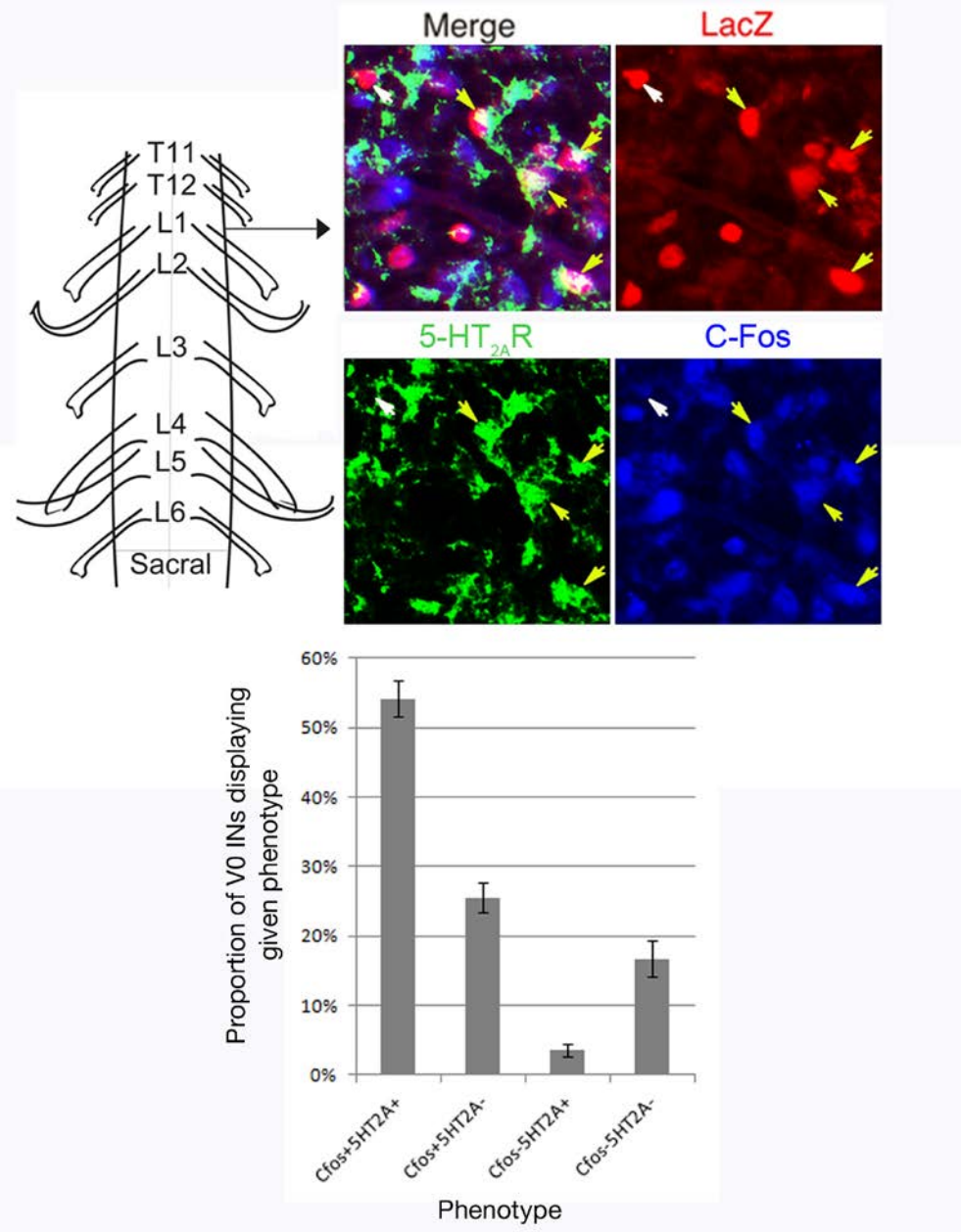

Figure 18: V0 cells that are active during locomotion express 5-HT$_{2A}$ receptors. Top Panels: Photomicrographs (40x magnification) taken from lamina VIII of neonatal *Dbx1*$^{LacZ/+}$ mouse lumbar spinal cord (coronal sections) that has undergone 1 hour of fictive locomotion, stained with antibodies for β-Gal (Red), 5-HT$_{2A}$ receptor (Green), and c-Fos (Blue). V0 cells that are active during locomotion (ie. colabel for c-Fos) and also colabel for 5-HT$_{2A}$ receptors are indicated by yellow arrows. V0 cells that lack c-Fos labelling and also lack 5-HT$_{2A}$ receptors are indicated by white arrows. **Bottom Panel:** Histogram showing the relative proportions of V0 INs displaying each of the four possible phenotypes.

Discussion

The mammalian central nervous system is a complex array of cells with varied morphology, connectivity, and function. In the spinal cord, the process of assigning functions to specific cell types has been made difficult by a relative inability to discriminate between one neuron and the next, a process that can be aided by molecular markers that correlate to a specific cell type and function. Transcription factors that are expressed early in embryonic development have the potential to serve as such markers, as they define neuronal characteristics such as morphology, migration pattern, connectivity, and neurotransmitter phenotype (Pillai *et al.* 2007). Thus there is a strong connection between transcription factor expression and neuronal function. Recently, transgenic mouse models have been developed that allow individual, genetically-defined interneuronal populations to be marked and visualized in the embryo, and followed into neonatal stages (Dougherty *et al.* 2010; Lanuza *et al.* 2004; Gosgnach *et al.* 2006; Pierani *et al.* 2001; Sapir *et al.* 2004; Zagoraiou *et al.* 2009; Zhang *et al.* 2008; Zhong *et al.* 2010). Accordingly, the process of characterizing these cell populations and assigning them specific functions in behaviours such as locomotion has begun (Lanuza *et al.* 2004; Gosgnach *et al.* 2006; Crone *et al.* 2008; Zhang *et al.* 2008).

In the present study, the V0 interneuron population was considered in terms of its potential function in the initiation of activity in the locomotor CPG. The V0 interneurons appear to be good candidates based on their location in lamina VIII of the lumbar spinal cord, the region known to be most critical to the production of the locomotor rhythm. They also receive projections from the brainstem

reticular formation. Previous work had demonstrated that the V0 population is a component of the locomotor CPG, as the targeted genetic ablation of the V0 cells results in a loss of left-right alternation during fictive locomotion. In considering this result, one might choose to dismiss the V0 interneurons as candidates for taking part in such a critical function as signalling the initiation of locomotion. If locomotor activity, albeit abnormal, can still be initiated in their absence, how can the V0 interneurons be responsible for its initiation? In considering this question, it is important to consider that this function may not be the sole responsibility of a single neuronal population. It is entirely possible that the initiation of locomotion is a process that is triggered by more than one population of interneurons working together, such that a subpopulation of the V0 cells might work together with one or more other neuronal populations to initiate locomotor activity in the spinal cord. If this is the case, the loss of any one population (i.e. the V0 cells) may be insufficient to stop the initiation of locomotion. The remaining populations may compensate for the lost population or even "take over" its function. Certainly this seems possible when one considers that each of the genetically-defined interneuron populations of the ventral horn (V0-V3) have been selectively silenced or ablated (Lanuza *et al.* 2004; Gosgnach *et al.* 2006; Crone *et al.* 2008; Zhang *et al.* 2008), and in all cases rhythmic locomotor activity persists.

Here, the potential role for the V0 cells in the initiation of locomotion was analyzed by evaluating the V0 cells in terms of specific criteria that would be requirements of first-order interneurons of the locomotor CPG. First I investigated whether these cells receive monosynaptic connections from the reticular

formation, the nucleus that is known to signal the initiation of locomotor CPG activity. In the DiI transport experiments detailed above, substantial evidence has been provided to indicate that V0 interneurons do indeed receive such monosynaptic innervation. It is notable that although only 32% of the observed putative "synapses" (ie. DiI-labeled punctae that co-labeled for synaptotagmin) were reported to connect onto V0 interneurons, this proportion could potentially underreport the frequency of said connectivity, as it considers only cases in which DiI^{+}, $synaptotagmin^{+}$ 'synapses' were observed in close proximity to V0 cell nuclei. Because the *Dbx1*$^{LacZ/+}$ transgenic mouse line expresses the β-Gal reporter in the nucleus only, the full dendritic arbors of the V0 interneurons could not be visualized. Thus it is likely that a proportion of the DiI^{+}, $synaptotagmin^{+}$ synapses that were observed to be 'in the absence' of a V0 cell may in fact have synapsed on to a V0 dendrite, rather than another cell type altogether. Nonetheless, it does seem clear that other cell types also receive innervation from the reticular formation, supporting our suggestion that the initiation of locomotion may indeed be a function that is shared between more than one cell type. Finally, the fact that the reticular formation also plays a key role in postural control (Drew *et al.* 2004) cannot be overlooked. It is therefore possible that some of the labeled reticular neurons were not necessarily locomotor-related. Despite this, it can certainly be concluded that the V0 interneurons meet the initial criteria for first-order interneurons of the locomotor CPG: they receive monosynaptic connections from the brainstem nuclei responsible for signaling the initiation of locomotion.

A second requirement for potential first-order interneurons of the locomotor

CPG is a sensitivity to 5-HT, and specifically the expression of both the 5-HT_7 (especially in tissue rostral to L3) and 5-HT_{2A} (especially in tissue caudal to L2) receptors. The data obtained from the antibody stains described within provide evidence that a significant proportion of V0 interneurons express each of these 5-HT receptor subtypes. Triple-labeling experiments which followed fictive locomotor activity demonstrated that a majority of V0 cells that expressed c-Fos also expressed 5-HT_7 and 5-HT_{2A} receptor subtypes. It was also observed that V0 cells that did not express c-Fos were unlikely to express the 5-HT_7 and 5-HT_{2A} receptor subtypes. These data indicate a clear link between the expression of 5-HT_7 and 5-HT_{2A} receptors by V0 cells, and their involvement in locomotor activity.

It is notable that the proportion of V0 interneurons expressing 5-HT_7 receptors was distributed in a rostral-caudal gradient, with a greater number of V0 cells expressing 5-HT_7 receptors in the rostral-most CPG segments (T12) and significantly fewer 5-HT_7 receptors on V0 cells located in L5 and L6. This is interesting for two reasons. First, it matches well with the rostral-caudal gradient previously described for the rhythmogenic capacity of the locomotor CPG (Kjaerulff and Kiehn, 1996), thus pointing towards a critical role for the V0 interneurons in the production of the rhythm. Second, since it has been demonstrated that the application of 5-HT_7 receptor antagonists to the rostral spinal cord blocks fictive locomotor activity (Liu and Jordan, 2005), it is possible that this effect may be mediated by actions on V0 interneurons.

Taken together, the experiments detailed herein provide support for the hypothesis that the V0 interneuron population may include first-order

interneurons of the locomotor CPG that play a role in initiating locomotor activity in the spinal cord. Further evidence is certainly needed to confirm this role. I suggest that cell-specific gene expression analysis should be used to assist our understanding of cell function. Single-cell RT-PCR procedures have the potential to provide information on gene expression at the level of a single neuron, such that individual cells may be further classified into subpopulations based on more specific patterns of expression of key genes that play a role in defining cell function, such as genes that encode neurotransmitter phenotype, receptors (such as 5-HT_7 and 5-HT_{2A} receptors), and additional transcription factors. While antibody stains such as those described in this thesis can provide some information on proteins expressed by specific cell populations, these types of experiments are limited in the number of proteins that may be labeled in a given experiment (based on the limited number of fluorophores available, and the number of wavelengths of light that may be observed by confocal fluorescence microscope technology). This makes it difficult to identify the host of proteins expressed by each cell, and thus correlate patterns of coexpression for more than a few proteins at a time. Single-cell RT-PCR has the potential to reveal information on multiple genes for a given cell, providing detailed information on patterns of gene coexpression that cannot be provided by immunohistochemistry. In addition, single-cell RT-PCR would allow us to identify a population of excitatory V0 interneurons, as the mRNAs encoding reporters for excitatory (ie. VGlut2) are present in the nucleus despite the fact that these proteins can only be detected in the axon terminals. Once the excitatory subpopulation of V0 interneurons is

isolated, molecular genetic tools can be incorporated to target these cells and selectively ablate, silence, or activate functionally specific subpopulations of interneurons in order to determine their specific function during motor behaviour

The experiments detailed herein represent a first step towards determining the role of the V0 interneurons in the generation of rhythmic locomotor activity. As details about each cell type of the mammalian spinal cord emerge, the field of neuroscience looks toward a future in which a complex map has been constructed, with specific interneurons fitted together like pieces of a puzzle, and connectivity, electrophysiological properties, neurotransmitter and receptor phenotypes, and ultimately function assigned to each.

Bibliography

Alcedo, J., Ayzenozon, M., Von Ohlen, T., Noll, M., Hooper, J.E. (1996). The *Drosophila smoothened* gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* **86**: 221.

Alexandre, C., Jacinto, A., Ingham, P.W. (1996). Transcriptional activation of hedgehog target genes in *Drosophila* is mediated directly by the Cubitus interruptus protein, a member of the GLI family of zing finger DNA-binding proteins. *Genes Dev.* **10**: 2003-2013.

Alvarez, F.J., Dewey, D.E., McMillin, P., Fyffe, R.E.W. (1999). Distribution of cholinergic contacts on Renshaw cells in the rat spinal cord: a light microscopic study. *J. Physiol*. **515**:787–797.

Armstrong, R.C., and Montminy, M.R. (1993). Transsynaptic control of gene expression. *Rev. Neurosci.* **16**: 17-29.

Arvidsson, U., Ulfhake, B., Cullheim, S., Ramirez, V., Shupliakov, O., Hokfelt, T. (1992). Distribution of calbindin D28k-like immunoreactivity (LI) in the monkey ventral horn: do Renshaw cells contain calbindin D28k-LI? *J. Neurosci.* **12**:718–728.

Andén, N.E., Jukes, M.G.M., Lundberg, A., Vyklický, L. (1966a). The effects of DOPA on the spinal cord. 1. Influence on transmission from primary afferents. *Acta Physiol. Scand.* **67**: 373-386.

Andén, N.E., Jukes, M.G.M., Lundberg, A. (1966b). The effects of DOPA on the spinal cord. 2. A pharmacological analysis. *Acta Physiol. Scand.* **67**: 387-397.

Baev, K.V., Beresovskii, V.K., Kebkalo, T.G., and Savoskina, L.A. (1988). Afferent and efferent connections of brainstem locomotor regions study by means of horseradish peroxidase transport technique. *Neuroscience* **26**: 871-892.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T.M., Rubenstein, J.L.R., Ericson, J. (1999). Homeobox gene *Nkx2.2* and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**: 622-627.

Burill, J.D., Moran, L., Goulding, M.D., Saueressig, H. (1997). PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1 interneurons that require PAX6 for their development. *Development* **124**: 4493-4503.

Butt, S.J.B., Lebret, J.M., Kiehn, O. (2002). Organization of left-right coordination in the mammalian locomotor network. *Brain Res. Rev.* **40**: 107-117.

Carlsson, A., Lindqvist, M., Magnusson, T., Waldeck, B. (1958). On the presence of 3-hydroxytyramine in the brain. *Science* **127**: 141.

Carlsson, A., Magnusson, T., Rosengren, E. (1963). 5-hydroxytryptamine of the spinal cord normally and after transection. *Experimentia* (Basel) **19**: 359-360.

Carlsson, A., Falck, B., Fuxe, K., Hillarp, N.-Å. (1964). Cellular localization of monoamines in the spinal cord. *Acta Physiol. Scand.* **60**: 112-119.

Carr, P., Huang, A., Noga, B., Dai, X., Jordan, L.M. (1995). Cytochemical characterization of cat spinal neurons activated during fictive locomotion. *Brain Res. Bull*. **37**: 213–218.

Chen, Y., and Stuhl, G. (1996). Dual roles for *patched* in sequestering and trasducing Hedgehog. *Cell* **87**: 553.

Clarac, F. (2008). Some historical reflections on the neural control of locomotion. *Brain Res. Rev.* **57**: 13-21.

Cowley, K.C., and Schmidt, B.J. (1994). A comparison of motor patterns induced by N-methyl-D-aspartate, acetylcholine and serotonin in the in vitro neonatal rat spinal cord. *Neurosci. Lett.* **171**: 147-150.

Cowley, K.C., and Schmidt, B.J. (1995). Effects of inhibitory amino acid antagonists on reciprocal inhibitory interactions during rhythmic motor activity in the in vitro neonatal rat spinal cord. *J. Neurophysiol.* **74**: 1109-1117.

Cowley, K.C., and Schmidt, B.J. (1997). Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. *J. Neurophysiol.* **77**: 247-259.

Crone, S.A., Quinlan, K.A., Zagoraiou, L., Droho, S., Restrepo, C.E., Lundfald, L., Endo, T., Setlak, J., Jessell, T.M., Kiehn, O., Sharma, K. (2008). Genetic ablation of V2a ipsilateral interneurons disrupts left-right locomotor coordination in mammalian spinal cord. *Neuron* **60**: 70-83.

Crone, S.A., Zhong, G., Harris-Warrick, R., Sharma, K. (2009). In mice lacking V2a interneurons, gait depends on speed of locomotion. *J. Neurosci.* **29**(21): 7098-7109.

Cullheim, S., Kellerth, J.O. (1982). Two kinds of recurrent inhibition of cat spinal α-motoneurones as differentiated pharmacologically. *J. Physiol.* **312**:209–224.

Dahlström, A., and Fuxe, K. (1965). Evidence for the existence of monamine neurons in the central nervous system. II. Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron system. *Acta Physiol. Scand.* **64**: Suppl. 247.

Dai, X., Douglas, J.R., Nagy, B., Noga, B.R., Jordan, L.M. (1990). Localization of spinal neurons activated during treadmill locomotion using the c-Fos immunohistochemical method. *Soc. Neurosci. Abstr.* **16**: 889.

Dai, X., Douglas, J.R., Noga, B.R., Jordan, L.M. (2005). Localization of spinal neurons activated during locomotion using the c-Fos immunohistochemical method. *J. Neurophysiol.* **93**(6): 3445-3452.

Devore, J., and Peck, R. (2001). *Statistics: The Exploration and Analysis of Data, Fourth Edition.* Pacific Grove: Brooks/Cole.

Dougherty, K.J., Kiehn, O. (2010). Firing and cellular properties of V2a interneurons in the rodent spinal cord. *J. Neurosci.* **30**(1): 24-37.

Drew, T., Prentice, S., and Schepens, B. (2004). Cortical and brainstem control of locomotion. *Prog. Brain Res.* **143**: 251-261.

Eccles, J.C., Fatt, P., Koketsu, K. (1954). Cholinergic and inhibitory synapses in a path from motor-axon collaterals to motorneurones. *J. Physiol.* **126**:524–562.

Echelard, Y., Epstein, D. J., St..Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jeesell, T. M., and Edlund, T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning along the rostrocaudal axis of the neural tube. *Cell* **81**: 747-756.

Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V., Jessell, T.M. (1997). Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. *Cold Spring Harbor Symp. Quant. Biol.* **62**: 451-466.

Flourens, M.-J.-P. (1824). Recherches Expérimentales sur les propriétés et les Fonctions du Système Nerveux, dans les Animaux Vertébrés (Experimental Studies on the Properties and Functions of the Nervous System in Vertebrate Animals). Chez Crevot, Paris.

Freusberg, A. (1874). Reflexbewegungen beim Hunde (Reflex movements in dogs). *Pflügers Arch. Ges. Physiol.* **9**: 358–391.

Fritsch, G.T., Hitzig, E. (1870). On the electrical excitability of the cerebrum *In*: Von Bonin, G. (1960). trans., *Some Papers on the Cerebral Cortex.* Springfield IL, Charles C. Thomas.

Garcia-Campmany, L., Stam, F.J., and Goulding, M. (2010). From circuits to behaviour: motor networks in mammals. *Curr. Opin. Neurobiol.* **20**: 116-125.

Goulding, M., Lanuza, G., Sapir, T., Narayan, S. (2002). The formation of sensorimotor circuits. *Curr. Opin. Neurobiol.* **12**(5): 508-515.

Graham Brown, T.G. (1911). The intrinsic factors in the act of progression in the mammal. *Proc. Roy. Soc. London B: Biology* **84**: 308–319.

Graham Brown, T.G. (1914). On the fundamental activity of the nervous centres: together with an analysis of the conditioning of rhythmic activity in progression, and a theory of the evolution of function in the nervous system. *J. Physiol.* **48**: 18–41.

Graham Brown, T. (1915). On the activities of the central nervous system of the unborn foetus of the cat, with a discussion of the question whether progression (walking, etc.) is a 'learnt' complex. *J. Physiol.* (London) **49**, 208–215.

Grillner, S. (1969). The influence of DOPA on the static and the dynamic fusimotor activity to the triceps surae of the spinal cat. *Acta Physiol. Scand.* **77**: 490–509.

Grillner, S., and Zangger, P. (1979). On the central generation of locomotion in the low spinal cat. *Exp. Brain Res.* **34**: 241–261.

Grillner, S. Control of locomotion in bipeds, tetrapods, and fish (1981). In: Brookhart, J.M., Mountcastle, V.B. (Eds.) *Handbook of Physiology - The Nervous System - Motor control.* Bethesda, MD: American Physiological Society, sect. 1, vol. II, p. 1179-1236.

Gross, M.K., Moran-Rivard, L., Velasquez, T., Nakatsu, M.N., Jagla, K.,

Goulding, M. (2000). Lbx1 is required for muscle precursor migration along a lateral pathway into the limb. *Dev.* **127**(2): 413-424.

Grossman, K.S., Giraudin, A., Britz, O., Zhang, J., Goulding, M. (2010). Genetic dissection of rhythmic motor networks in mice. *Prog. Brain Res.* **187**: 19-37.

Higashijima, S., Masino, M.A., Mandel, G., Fetcho, J.R. (2004). Engrailed-1 expression marks a primitive class of inhibitory spinal interneuron. *J. Neurosci.* **24**(25): 5827-5839.

Hochman, S. (2007). Spinal cord. *Curr. Biol.* **17**(22): 950-955.

Hodgkin, A.L., Huxley, A.F., Katz, B. (1952). Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. *J. Physiol*. **115**(4): 424-448.

Hodgkin, A.L., and Huxley, A.F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol*. **117**(4): 500-544.

Holmqvist, B.I., Östholm, T., Ekström, P. (1991). DiI tracing in combination with immunocytochemistry for analysis of connectivities and chemoarchitectonics of specific neural systems in a teleost, the Atlantic salmon. *J. Neurosci. Methods* **42**: 45-63.

Huang, A., Noga, B.R., Carr, P., Fedirchuk, B., Jordan, L.M. (2000). Spinal cholinergic neurons activated during locomotion: immunohistochemical localization and electrophysiological characterization. *J. Neurophysiol.* **83**: 3537–3547.

Hultborn, H., Jankowska, E., Lindstrom, S. (1971). Recurrent inhibition from motor axon collaterals of transmission in the Ia inhibitory pathway to motoneurones. *J. Physiol*. **215**:591–612.

Illert, M., Jankowska, E., Lundberg, A., Odutola, A. (1981). Integration in descending motor pathways controlling the forelimb in the cat. 7. Effects from the reticular formation on C3-C4 propriospinal neurones. *Exp. Brain. Res.* **42**(3-4):269-81.

Jankowska, E., Jukes,M.G.M., Lund, S., Lundberg,A. (1967a). The effect of DOPA on the spinal cord: V. Reciprocal organization of pathways transmitting excitatory action on alphamotoneurones of flexors and extensors. *Acta Physiol. Scand*. **70**: 369–388.

Jankowska, E., Jukes, M.G.M., Lund, S., Lundberg, A. (1967b). The effect of DOPA on the spinal cord: VI. Half-centre organization of interneurones transmitting effects from the flexor reflex afferents. *Acta Physiol. Scand.* **70**: 389–402.

Jankowska, E., and Lundberg, A. (1981). Interneurons in the spinal cord. *Trends Neurosci*. **4**: 230–233.

Jasmin, L., Gogas, K.R., Ahlgren, S.C., Levinet, J.D., Basbaum, A.I. (1994). Walking evokes a distinctive pattern of fos-like immunoreactivity in the caudal brainstem and spinal cord of the rat. *Neurosci.* **58**: 275-286.

Karunaratne, A., Hargrave, M., Poh, A., Yamada, T. (2002). GATA proteins identify a novel ventral interneuron subclass in the developing chick spinal cord. *Dev. Biol*. **249**: 30–43.

Kiehn, O. (2010). Development and functional organization of spinal locomotor circuits. *Curr. Opin. Neurobiol.* doi:10.1016/j.conb.2010.09.004

Kiehn, O., and Kjaerulff, O. (1998). Distribution of central pattern generators for rhythmic motor outputs in the spinal cord of limbed vertebrates. *Ann. NY Acad. Sci.* **860**: 110-129.

Kjaerulff, O., Barajon, I., Kiehn, O. (1994). Sulphorhodamine-labelled cells in the neonatal rat spinal cord following chemically induced locomotor activity *in vitro. J. Physiol.* **478**: 265-273.

Kjaerulff, O., and Kiehn, O. (1996). Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord In Vitro: A lesion study. *J. Neurosci.* **16**: 5777-5794.

Kjaerulff, O., and Kiehn, O. (1997). Crossed rhythmic synaptic input to motoneurons during selective application of the contralateral spinal locomotor network. *J. Neurosci.* **17**: 9433–9447.

Köbbert, C., Apps, R., Bechmann, I., Lanciego, J.L., Mey, J., Thanos, S. (2000). Current concepts in anatomical tracing. Prog. Neurobiol. **62**: 327-351.

Krauss, S., Concordet, J.-P., and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene Shh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**: 1431-1444.

Kudo, N., and Yamada, T. (1987). N-methyl-D,L-aspartate-induced locomotor activity in a spinal cord-hindlimb muscles preparation of the newborn rat studied in vitro. *Neurosci. Letters* 75 (1): 43-48.

Lafreniere-Roula, M., and McCrea, D. (2005). Deletions of rhythmic motor neuron activity during fictive locomotion and scratch provide clues to the organization of the mammalian central pattern generator. *J. Neurophysiol.* **94**: 1120-1132.

Lanuza, G., Gosgnach, S., Pierani, A., Jessel, T., and Goulding, M. (2004). Genetic identification of spinal interneurons that coordinate left-right locomotor activity necessary for walking movements. *Neuron* **42**: 375-386.

Li, W.C., Higashijima, S., Parry, D.M., Roberts, A., Soffe, S.R. (2004). Primitive roles for inhibitory interneurons in developing frog spinal cord. *J. Neurosci.* **24**: 5840-5848.

Ling, G., and Gerard, R.W. (1949). The normal membrane potential of frog sartorius fibers. *J. Cell Physiol.* **34**(3): 383-396.

Liu, J., and Jordan, L.M. (2005). Stimulation of the parapyramidal region of the neonatal rat brainstem produces locomotor-like rhythmic activity involving spinal 5-HT_7 and 5-HT_{2A} receptors. *J. Neurophysiol*. **94**: 1392-1404.

Lundberg, A. (1981). Half-centres revisited. In: Szentagotheu, J., Palkovits, M., Hamori, J. (Eds.), Regulatory Functions of the CNS.Motion and Organization Principles. Pergamon Akademiai Kiado, *Adv. Physiol. Sci.*, Budapest, Hungary, pp. 155–167.

Marigo, V., Davey, R.A., Zuo, Y., Cunningham, J.M., Tabin, C.J. (1996). Biochemical evidence that patched is the Hedgehog receptor. *Nature* **384**: 176.

Marmot, G. (1949). Studies on the axon membrane; a new method. *J. Cell Physiol*. **34**: 351-382.

Marti, E., Bumcrot, D. A., Takada, R., and McMahon, A. P. (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**: 322-325.

Matise, M.P., Joyner, A.L. (1997). Expression patterns of developmental control genes in normal and *Engrailed-1* mutant mouse spinal cord reveal early diversity in developing interneurons. *J. Neurosci.* **17**: 7805-7816.

Matsuyama, K., Mori, F., Nakajima, K., Drew, T., Aoki, M., Mori, S. (2004). Locomotor role of The corticoreticular-reticulospinal-spinal interneuronal system. *Prog. Brain. Res.* **143**: 239-249.

Munglani, R., and Hunt, S.P. (1995). Proto-ocogenes: basic concepts and stimulation induced changes in the spinal cord. *Prog. Brain Res.* **104**: 283-298.

McCrea, D.A. (2008). Organization of mammalian locomotor rhythm and pattern generation. *Brain Res. Rev.* **57**: 134-146.

Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M.K., Burrill, J., Goulding, M. (2001). *Evx1* is a postmitotic determinant of V0 interneuron identity in the spinal cord. *Neuron* **29**: 385-399.

Noga, B.R., Kriellaars, D.J., Brownstone, R.M., Jordan, L.M. (2003). Mechanism for activation of locomotor centers in the spinal cord by stimulation of the mesencephalic locomotor region. *J. Neurophysiol*. **90**(3): 1464-1478.

Orlovsky, G.N. (1969). Spontaneous and induced locomotion of the thalamic cat. *Biophysics* **14**: 1154-1162.

Orlovsky, G.N., Deliagina, T.G., and Grillner, S. Neuronal control of locomotion. New York: Oxford University Press (1999).

Peng, C.-Y., Yajima, H., Burns, C.E., Zon, L.I., Sisodia, S.S., Pfaff, S.L., Sharma, K. (2007). Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron* **53**: 813–827.

Peterson, B.W., Maunz, R.A., Pitts, N.G., Mackel, R.G. (1975). Patterns of projection and branching of reticulospinal neurons. *Exp. Brain Res.* **23**: 333-351.

Pierani, A., Brenner-Morton, S., Chiang, C., Jessell, T.M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**: 903–915.

Pierani, A., Moran-Rivard, L., Sunshine, M.J., Littman, D.R., Goulding, M., Jessell, T.M. (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. *Neuron* **29**: 367-384.

Pillai, A., Mansouri, A., Behringer, R., Westphal, H., Goulding, M. (2007). Lhx1 and Lhx5 maintain the inhibitory-neurotransmitter status of interneurons in the dorsal spinal cord. *Development* **134**(2): 357-366.

Philippson, M. (1905). L'autonomie et la centralisation dans le système nerveux des animaux (Autonomy and centralization in the animal nervous system). *Trav. Lab. Physiol. Inst. Solvay* (Bruxelles) **7**: 1–208.

Renshaw, B. (1946). Central effects of centripetal impulses in axons of spinal ventral roots. *J. Neurophysiol.* **9**: 191-204.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruizi, I., Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T., and Dodd, J. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**: 761-775.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A., and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of Sonic hedgehog autoproteolysis. *Cell* **81**: 445-455.

Rybak, I.A., Shevtsova, N.A., Lafreniere-Roula, M., McCrea, D.A. (2006-a). Modelling spinal circuitry involved in locomotor pattern generation: insights from deletions during fictive locomotion. *J. Physiol.* **577**: 617–639.

Rybak, I.A., Stecina, K., Shevtsova, N.A., McCrea, D.A. (2006-b). Modelling spinal circuitry involved in locomotor pattern generation: insights from the effects of afferent stimulation. *J. Physiol.* **577**: 641–658.

Sapir, T., Geiman, E.J., Wang, Z., Velasquez, T., Mitsui, S., Yoshihara, Y., Frank, E., Alvarez, F.J., Goulding, M. (2004). *Pax6* and *Engrailed-1* regulate two different classes of Renshaw cell development. *J. Neurosci.* **24**(5): 1255-1264.

Saueressig, H., Burrill, J., Goulding, M. (1999). Engrailed-1 and Netrin-1 regulate axon pathfinding by association interneurons that project to motor neurons. *Development* **126**: 4201-4212.

Schneider, S.P., Fyffe, R.E.W. (1992). Involvement of GABA and glycine in recurrent inhibition of spinal motor neurons. *J. Neurophysiol.* **68**:397–406.

Shefchyk, S.J., Jell, R.M., Jordan, L.M. (1984). Reversible cooling of the brainstem reveals areas required for mesencephalic locomotor region evoked treadmill locomotion. *Exp. Brain Res.* **56**(2): 257-262.

Sherrington, C.S. (1910). The Flexion-reflex of the limb, crossed extension-reflex, and reflex stepping and standing. *J. Physiol.* **40**(1-2): 28-121.

Shik, M. L., Severin, F. V., Orlovsky, G. N. (1966a). Control of walking and running by means of electrical stimulation of the mid-brain. *Biophvsics* **11**: 756-765.

Shik, M. L., Orlovsky, G. N., Severin, F. V. (1966b). Organization of locomotor synergism. *Biophysics* **11**: 1011–1019.

Smith, J.C., Feldman, J.L. (1987). In vitro brainstem-spinal cord preparations for study of motor systems for mammalian respiration and locomotion. *J. Neurosci.Methods* **21**(2-4): 321-333.

Smith, E., Hargrave, M., Yamada, T., Begley, C.G., Little, M.H. (2002). Coexpression of SCL and GATA3 in the V2 interneurons of the developing mouse spinal cord. *Dev. Dyn*. **224**: 231–237.

Sparks, D.L., Lue, L-F., Martin, T.A., Rogers, J. (2000). Neuronal tract-tracing using Di-I: A review and a new method to make make fast Di-I faster in human brain. *J. Neurosci. Methods* **103**: 3-10.

Stepien, A.E., and Arber, S. (2008). Probing the locomotor conundrum: descending the 'V' interneuron ladder. *Neuron* **60:** 1-4.

Stone, D.M., Hynes, M., Armanini, M., Swanson, T.A., Gu, Q., Johnson, R.L., Scott, M.P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J.E., de Sauvage, F., Rosenthal, A. (1996). The tumor-supressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384**: 129.

Stuart, D.G., and Hultborn, H. (2008). Thomas Graham Brown (1882–1965), Anders Lundberg (1920–), and the neural control of stepping. *Brain Res. Rev*. **59**: 74-95.

Swift, M.J., Crago, P.E., Grill, W.M. (2004). Applied electric fields accelerate the diffusion rate and increase the diffusion distance of DiI in fixed tissue. *J. Neurosci. Methods* **141**: 155-163.

Tanabe, Y., Roelink, H., and Jessell, T. M. (1995). Induction of motor neurons by Sonic hedgehog is independent of floor plate differentiation. *Curr. Biol*. **5**: 651-658.

Udenfriend, S., Weissbach, H., Bogdanski, D.F. (1957). Increase in tissue serotonin following administration of its precursor, 5-hydroxytryptophan. *J. Biol. Chem.* **224**: 803-810.

Unzer, J. (1771). Erste Gründe einer Physiologie der eigentlichen thierischen Natur thierischer Körper (The Principles of Physiology). Weidmanns, Erben and Reich, Leipzig.

Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A, Mendelson, M., Sander, M., Jessell,

T.M., Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to *Nkx6* genes in motor neuron and interneuron specification. *Neuron* **31**: 743-755.

van den Heuvel, M., and Ingham, P.W. (1996). *Smoothened* encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature* **382**: 547.

Walberg, F., Pompeiano, O., Westrum, L.E., and Hauglie-Hanssen, E. (1962). Fastigoreticular fibres in the cat. An experimental study with the silver method. *J. Comp. Neurol.* **119:** 187-199.

Wilson, D.M., and Wyman, R.J. (1965). Motor output patterns during random and rhythmic stimulation of locust thoracic ganglia. *Biophys. J.* **5**: 121–143.

Zhong, G., Droho, S., Crone, S., Dietz, S., Kwan, A.C., Webb, W.W., Sharma, K., Harris-Warrick, R.M. (2010). Electrophysiological characterization of the V2a interneurons and their locomotor-related activity in the neonatal mouse spinal cord. *J. Neurosci.* **30**(1): 170-182.

Zhou, Y., Yamamoto, M., Engel, J.D. (2000). GATA2 is required for the generation of V2 interneurons. *Development* **127**: 3829–3838.