2011

V1-Derived Renshaw Cells and IA Inhibitory Interneurons Differentiate Early During Development

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V1-DERIVED RENSHAW CELLS AND IA INHIBITORY INTERNEURONS DIFFERENTIATE EARLY DURING DEVELOPMENT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By

ANA BENITO GONZÁLEZ
B.S., Universidad Complutense de Madrid, 2005

2011
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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY ANA BENITO GONZÁLEZ ENTITLED V1-DERIVED RENSHAW CELLS AND IA INHIBITORY INTERNEURONS DIFFERENTIATE EARLY DURING DEVELOPMENT BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT

Benito González, Ana. Ph.D., Biomedical Sciences Program, Wright State University, 2011. V1-derived Renshaw Cells and Ia Inhibitory Interneurons Differentiate Early During Embryonic Development.

Locomotor development is dependent on the maturation of spinal cord circuits controlling motor output, but little is known about the development of the spinal interneurons that control motoneuron activity. This study focused on the development of Renshaw cells (RCs) and Ia inhibitory interneurons (IaINs), which mediate recurrent and reciprocal inhibition, respectively, two basic inhibitory circuits for motoneuron control. Both interneurons originate from the same progenitor pool (p1) giving rise to ventral spinal embryonic interneurons denominated V1. V1-derived interneurons (V1-INs) establish local inhibitory connections with ipsilateral motoneurons and express the transcription factor engrailed-1. This characteristic permitted the generation of transgenic mice that were used in this study to genetically label V1 interneuron lineages from embryo to adult. Adult V1-derived Renshaw cells and IaINs share some similar properties, both being inhibitory and establishing ipsilateral connections; but differ in morphology, location in relation to motor pools, expression of calcium binding proteins (calbindin vs. parvabumin), synaptic connectivity and function. These differences are already present in neonates, therefore the purpose of this study was to determine possible embryonic differentiation mechanisms.

Using 5’-bromodeoxyuridine birth-dating we demonstrated that V1-INs can be divided into early and late born groups. The early group quickly upregulates calbindin
expression and includes the Renshaw cells, which maintain calbindin expression through life. The second group includes many cells that postnatally upregulate parvalbumin, including IaINs. This later born group is characterized by upregulation of the transcription factor FoxP2 as they start to differentiate and is retained up to the first postnatal week in many V1-derived IaINs. In contrast, Renshaw cells express the transcription factor MafB that seems relatively specific to them within the V1-INs. Furthermore, Renshaw cells appear attracted to the ventral root exit region and follow a unique migratory route to become specifically placed at this location. In contrast, other V1 interneurons settle more medially and far from the ventral root exit region. MafB expression is upregulated in Renshaw cells only after they have reached their final position among motor axons. Therefore, the specific migration of Renshaw cells might be responsible for their final differentiation and unique relationship with motor axons in adult.
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ACKNOWLEDGEMENTS

I would like to first thank my parents, Mari Carmen and Juan Carlos, and my brother Carlos, because without their encouragement and support I would not have made it. My parents have always encouraged me to pursue my dreams and lead me in the right direction by teaching me what is important in life. My brother has always encouraged me to be brave and take risks. He has made a better person, also more mature and independent. For that and much more I will always be grateful to them. Also I want to thank my aunt, Elena, because she always listens and has contributed to keep me sane in many ways. I want to also thank to all my friends for helping me find myself and encourage me to be the person I wanted to be. I am grateful for their friendship and all the many things we have lived and learn from together. I would like to also thank my boyfriend, James, which has supported me since I started my PhD. and helped me in every possible way. Thanks to him I found the courage to deal with everyday and never give up. I have learned many things from him, more than I could not possibly enumerate here. I also want to thank everyone at the A-21 lab at the Cajal Institute for giving me the opportunity to do science and help me find my way to pursue a scientific career, for that and for their patience teaching me how to do science I will always be thankful. I want send a special thanks to Dr. Juan A. De Carlos because we both know that without his help I would not be writing this today. Finally, I would like to thank Dr. Francisco Alvarez for giving me the opportunity to pursue a career in science by accepting me in his lab and teach me how to be a better scientist. He has always been able to transmit his passion for science and encourage me to follow my own path.
I. INTRODUCTION

Higher primates, including humans, lack locomotor skills at birth. This is contrary to other mammals, such as elephants and giraffes that quickly develop their body position, posture and ability to walk around the time of birth. It is therefore tempting to speculate that development of motor circuits in humans occurs postnatally through activity-dependent refinement mechanisms. Nevertheless, basic circuits of the spinal motor system seem preformed in embryo in all species. For example, recurrent and reciprocal inhibition, two fundamental circuits of motor control and that are the focus of this study, are present in newborn babies (Mc Donough et al., 2001). Although they also undergo significant postnatal maturation their presence at birth implies earlier specification of the basic elements that create these circuits. As will be explained below, motor circuit development is in fact not too different in species that start to walk at birth or days, weeks or months after birth. In both cases basic motor circuit elements likely develop in embryo through activity-independent mechanisms. This of course does not exclude posterior functional refinement of the preformed circuits.

Species with different onsets of walking after birth are categorized as altricial or precocial. Altricial refers to species that "require nourishment", meaning they are not able to walk at birth, while precocial are species that can walk at, or just after, birth. The significance of these differences has been discussed for many years. Recent data however suggest that motor system development is in fact a functional continuum among all species (Garwicz et al., 2009), and that there are no qualitative differences. The key is to measure onset of locomotion since conception and not from birth and
correct this for brain size. In this case brain size and time for full brain development emerge as the main factors that determine the onset of walking (Garwicz et al., 2009). Natural selection will tend to favor rapid development, especially during early stages associated with high mortality. Thus, a long postnatal period until motor maturity must be considered the price paid by some other evolutionary more advantageous feature, such as early birth or a larger more complex brain. Differences in postnatal maturation might not imply, however, major differences in the earlier process of basic motor circuit assembly.

Interestingly, some factors were identified that might delay the onset of walking (Garwicz et al., 2009). The most important one was adopting the plantigrade position characteristic of humans and rodents, and frequently associated with manipulative capacity in the upper extremities or forelimbs. In this sense, animals like ungulates and carnivores with simplified extremities adapted for fast locomotion might accelerate motor system development by losing circuit components that allow finer control of movement. In any case, the conclusions from Garwicz and colleagues imply that the major developmental milestones for the assembly of basic locomotor motor circuits are conserved in most mammalian species (altricial or precocial) and occur in embryo or postnatally (depending of time of birth and brain size). But in both cases it could be expected that they are similar processes, and thus, likely independent of activity-dependent refinement. In this thesis we will investigate early embryonic mechanisms for the assembly of key elements of inhibitory spinal motor circuits. These circuits are present at birth and later undergo postnatal maturation (Mc Donough et al., 2001).
Locomotion, as many behaviors fundamental to animal life, such as breathing, and chewing, are rhythmic activities controlled by neuronal networks located in different parts of the central nervous system. The central nervous system hierarchically controls movement, involving different structures, such as cortex, brainstem, spinal cord, cerebellum and basal ganglia. Each component provides different motor control features. The basic rhythmicity and patterning for stepping and other locomotion behaviors is produced by neuronal circuits contained entirely within the spinal cord. Little is known about how spinal cord circuits develop and how different types of neurons are generated, specified and incorporate themselves into these circuits. Understanding how and when cell specialization occurs and what factors are involved in the differentiation process are key for understanding the maturation of motor control and the abnormalities and dysfunctions originated during early development.

Within spinal cord circuits two types of neurons need to mature: motoneurons and interneurons (INs). Motoneurons generate the motor output to the muscle, while INs establish local circuits that control the activity of motoneurons. Past research has focused on motoneuron development (Jessell, 2000), recently, interest has turned to spinal cord INs. A major breakthrough was the finding that all adult spinal INs develop from a few embryonic subclasses; six dorsal (dl1, dl2, dl3, dl4, dl5, and dl6) and four ventral (V0, V1, V2, and V3), each derived from a progenitor domain located in a different dorso-ventral region of the proliferative neuroepithelium in the early neural tube (Goulding, 2009).

This study is concerned with the diversification of two subtypes of inhibitory INs, Renshaw cells (RCs) and Ia inhibitory interneurons (IaINs) from the V1
embryonic group. Renshaw cells and IaINs have different functional roles and connections in the adult. Renshaw cells are activated by inputs from motor axons and mediate recurrent inhibition by inhibiting homonymous and synergistic motoneurons (Eccles et al., 1954). IaINs receive inputs from Ia proprioceptive afferents and mediate reciprocal inhibition by inhibiting motoneurons in motor pools innervating muscles that are antagonists of the muscle of origin of the Ia afferent (Eccles et al., 1956).

These different INs express very different phenotypes not only in the adult but also in neonates. These include different connections, location and expression of calcium binding proteins (Alvarez et al., 2005; Siembab et al., 2010). Their advanced phenotypic differentiation in the newborn implies an earlier embryological program for their differentiation. Thus, the purpose of this study was to determine when these two groups of V1-derived INs start to differentiate from each other in embryo and generate some plausible hypothesis about their mechanisms of differentiation. We investigated the times at which these two types of V1-INs are generated ("birthdate") and start their differentiation, what transcription factors are specific to them and could direct their differentiation, and finally we analyzed their early migration pathways to provide some explanations for their different relationship with motor axons.
II. BACKGROUND

The main goal of this thesis is to better understand the development of interneurons (INs) in the motor circuits of the spinal cord ventral horn. Interneurons control motoneuron firing during locomotion and convey sensory feedback information to modulate the motor output; they also relay the descending commands that exert voluntary control of motor behaviors.

Overview of motor system and its development

The motor system is involved in the control and generation of voluntary and reflex movements. In order to do this, the motor system integrates motor commands generated in the central nervous system with ongoing sensory information to control the complex mechanical machinery of the musculoskeletal system. This is achieved by coordinating three levels of motor control: the spinal cord, the descending systems of the brainstem, and the interconnected motor areas of the cerebral cortex, cerebellum and basal ganglia. These different components are organized hierarchically and in parallel. The hierarchical organization enables higher centers to give relatively general commands without having to specify the details of the motor action. By means of their parallel organization the higher centers of the motor system can issue commands that can act directly on the spinal cord, the lowest level of the chain, or can affect spinal circuits indirectly through other parallel descending systems. For example, the corticospinal tract controls spinal circuit indirectly through motor pathways originated in brainstem nuclei (for example, rubrospinal and pontine and medullary reticulospinal projections) but, it also controls, directly, spinal INs and in some cases the
motoneurons. Direct monosynaptic corticospinal influence on spinal motoneurons is largely restricted to upper limb musculature involved in fine manipulation in human and non-human primates (Kuypers, 1962, 1964; Porter and Lemon, 1993). Initially, each of these areas develops independently and later become interconnected. Descending system connections with the spinal cord is the last step in the maturation of the motor system and is mostly a postnatal process manifested behaviorally mainly in the increased postural control with age of pups and babies (reviewed in Altman and Bayer, 2001; Vinay et al., 2005). The arrival of descending systems into the spinal cord has been proposed to result in the reorganization of spinal segmental systems (reviewed in Clowry, 2007), but initially the spinal cord circuits that control motor output develop in isolation. Self-organization of basic circuitry capable of generating patterned motor output is manifested in the isolated embryonic and neonatal spinal cord, which is capable of generating rhythmic motor output that resembles locomotion in that there is alternation between flexors and extensors and contralateral sides of the spinal cord (Smith and Feldman, 1987; reviewed in Whelan, 2003; Vinay et al., 2002; Clarac et al., 2004a, b).

The spinal cord is responsible for generating complex spatiotemporal patterns of motor output that result in organized muscle activation during reflexes and rhythmic motor patterns, such as locomotion. The spinal cord contains all necessary circuits to mediate a variety of automatic and stereotyped reflexes and also locomotion. At the beginning of the past century, Sherrington demonstrated that virtually all reflexes involve the integrated activation and inhibition of activity in different muscle groups, and these continue to function even if the cord is disconnected from the brain.
(Sherrington, 1910). He suggested that many of these actions are coordinated by spinal INs. Ultimately, all interneuronal controls converge on the motoneurons that innervate the skeletal muscles. To stress the importance of this convergence, Sherrington called the motoneurons the final common path (Sherrington, 1947). Almost simultaneously, Graham-Brown demonstrated that rhythmic locomotion can also be elicited in spinalized cats that are positioned on a moving treadmill with or without sensory afferents (Graham-Brown, 1911) and this lead to the proposition of spinal “centers” that are interconnected in such a manner as to produce alternating stepping movements (Graham-Brown, 1916). Nowadays, these spinal centers are called central pattern generators (CPGs) and are believed to generate and control the basic rhythmicity and pattern of motoneuron output during locomotion (Kiehn, 2006; Brownstone and Bui, 2010). Both, reflexes and CPGs, rely on local circuits formed by a large number of spinal INs. Historically the naming of spinal INs involved in reflex pathways occurred first (i.e., Ia inhibitory INs involved in reciprocal inhibition during monosynaptic stretch reflexes, and others like Ib/Ia inhibitory INs, group II INs… etc; Jankowska, 1992). However, spinal INs are functionally versatile and the same interneuron can be recruited by reflex pathways, descending systems and the spinal CPG to modulate motoneuron activity (reviewed in Jankowska, 2001; McCrea, 2001). These basic networks of INs are believed to outline the general principles of organization and function in the vertebrate locomotor system. Unfortunately many elements of these circuits are still unknown, for example the basic cellular components of the rhythmic core have not yet been characterized. A developmental perspective that focuses on their
initial development and wiring might generate important information not only about their origins but also about their basic organization.

**Basic organization of the spinal cord and its development**

**Rostro-caudal regions of the spinal cord**

The spinal cord is the part of the central nervous system that extends caudal to the medulla and is located within the vertebral column. It is longitudinally divided into cervical, thoracic, lumbar, and sacral segments (Fig.1). Each region controls the musculoskeletal system of a different part of the body. The cervical region controls the neck muscles, respiration and upper arms, the thoracic region the axial musculature, the lumbar region controls the hip and lower limbs and finally the sacral region controls several visceral sphincters (i.e., bladder) and the tail. As a result there is diversity in the motor pools located in each region, each controlled by different types of descending systems and interneuronal circuits.

Most of our studies are mainly focused on the ventral horn of the lumbar area, where the motor control of the lower limbs is organized. The lumbar spinal cord is divided into 5 segments in humans and 6 in mice. These can be grouped in lower lumbar segments (L4 through L6) that are biased towards extensor muscles and upper lumbar segments (L1 to L3) which are biased towards flexor muscles (Fig.1). There are also important differences in their capacity for rhythmic locomotion and usually upper lumbar segments are considered to be more excitable and readily available to start rhythmic motor output than lower segments (Kiehn, 2006; Bonnot and Morin, 1998; Bonnot, et al., 2002). Another difference is that upper lumbar segments contain the
Figure 1. Spinal column segmental divisions. Lumbar levels are further classified in upper lumbar (L1 and L2) biased towards flexor muscles and lower lumbar (L4 and L5) biased towards extensor muscles. Modified from Molander et al., 1984.
intermediolateral column of visceral motoneurons (preganglionic sympathetic neurons) that is continuous with thoracic segments and has its own independent control. These differences imply that the INs and circuits controlling motor output are not necessarily identical in upper and lower lumbar segments.

Lumbar development lags slightly behind cervical development. Neurogenesis and differentiation begins rostrally and progresses caudally in both rodents and humans. In the rat, most cervical motoneurons are generated between embryonic day 11 (E11) and E12 while most lumbar motoneurons appear between E12 and E13 (Barber et al., 1991; Altman and Bayer, 1974; note that in the mouse similar developmental stages are reached 24-36 hours earlier, see later). The same rostrocaudal pattern of cell generation was noted in dorsal root ganglion (DRG) neurons (at cervical levels most cells are generated at E11.5 and at E12.5 at lumbar levels; Lawson and Biscoe, 1979). Later, there is also a rostrocaudal progression in the functional maturation of locomotion due to the earlier development of descending connections in the cervical region compared to lumbar. For example, a report from Clarac et al. (1998) explains the early bias in neonatal rodents towards favoring forelimbs for initial crawling as a result of the lack of enough postural tonus provided by descending systems in the lumbar region controlling the hindlimbs. Nevertheless, both cervical and lumbar spinal cord have functional CPGs at birth and each can drive robust locomotion-like activity in both regions and also interact between them (Ballion et al., 2001; Gordon et al., 2008). Thus, neonatal rodents and humans can display coordinated locomotor activity between cervical and lumbar regions in situations in which weight-bearing postural control is not necessary, for example during air-stepping and swimming. In summary, there could
be developmental differences in between different rostrocaudal regions because of differences in motor pool composition and also because the existence of rostrocaudal gradients in neurogenesis and functional maturation.

**Dorso-ventral organization of the spinal cord.**

Besides rostro-caudal differences, the spinal cord is also organized dorso-ventrally. Transversally, the spinal cord is divided in all segments into two halves: the dorsal and ventral horns. The dorsal horn receives sensory inputs through the dorsal root and most dorsal horn neurons are functionally involved in the relay of cutaneous sensory information to higher centers in the brain or integrating proprioceptive or cutaneous input to be sent to the ventral horn (Fig.2). The ventral horn contains the motoneurons and all the INs that control their activity (Fig.2 and 3). Motor output is transmitted through the motor axons that exit through the ventral roots. Dorsal and ventral horns are divided in different laminae based on the size, morphology and density of neurons. Rexed (1952, 1954), divided the gray matter of the cat spinal cord into nine layers or laminae and a tenth region around the central canal. The ventral horn consists of laminae VI to IX. Lamina IX is defined as the region containing the motor pools; lamina VIII occupies the medial ventral horn and contains many large INs which send projections to the contralateral side of the spinal cord. The remainder of the ventral horn is defined as lamina VII and contains medium to small size INs, many of which are believed to form part of motor circuits. Finally, lamina VI is an ill-defined region between the ventral-most dorsal horn lamina V and lamina VII. This lamina occurs only in the cervical and lumbar enlargements, the regions that control the
Figure 2. Basic structure and organization of the spinal cord. Top, shows a diagram of the spinal cord in cross-section. Bottom, low magnification confocal image of a P40 mouse spinal cord transverse section immunostained for NeuN (red). This section corresponds to the lumbar 4 segment. NeuN is a neuronal marker of differentiated neurons. Neurons are contained within the gray matter (the core of the spinal cord) and are surrounded by white matter (unstained). The central canal is what remains from the original ventricle in the neural tube and is in the center of the spinal cord. The dotted line separates the gray matter from the outside white matter. The white matter contains all the axons from ascending, descending and propriospinal systems. Neuronal density is higher in the dorsal horn than in the ventral horn. The ventral horn is divided in basically three laminae: lamina IX containing the large NeuN-IR cell bodies of motoneurons; lamina VIII contains relatively large interneurons most of which are known to project to the contralateral side of the cord; lamina VII contains most of the interneurons that control the function of ipsilateral motoneurons.
Spinal cord

- Dorsal horn
- Lateral horn
- Grey matter
- Ventral horn
- White matter
- Dorsal root (Sensory input)
- Spinal ganglion
- Ventral root (Motor output)
- Central canal

P40

- Dorsal horn
- Central canal
- Ventral horn

NeuN
Figure 3. Distribution of NeuN-immunoreactive and choline acetyltransferase-immunoreactive neurons in spinal cord sections from lower thoracic to lower lumbar segments of the mouse spinal cord at postnatal day 15 (P15). Low magnification confocal images of spinal cord sections 50 μm thick. Motoneurons are labeled in lamina IX, using an antibody against Choline Acetyltransferase (left column, green neurons at the bottom of the section). All motoneurons and interneurons are labeled in red, using an antibody against NeuN that labels differentiated neurons (right column). A and B) Thoracic level; C and D) Lumbar 2; E and F) Lumbar 3; G and H) Lumbar 5; I and J) Lumbar 6. Note the different organization and cell numbers of motoneuron groups at different segmental levels.
extremities (Rexed, 1952 in cat; Molander et al., 1984; Molander et al., 1989 in rodents).

It has been known since the time of Cajal (Cajal, 1995) that at any particular spinal cord segment, neurogenesis always starts ventrally with the motoneurons and then proceeds dorsally generating different types of INs. Embryological studies pioneered by His (1886, reviewed in Altman and Bayer, 2001) distinguished between an alar and a basal plate based on the presence of an invagination in the ventricle, known as sulcus limitans. The alar plate gives rise to the dorsal horn and the basal plate to the ventral horn (Fig.4). The idea that motoneurons were generated ventrally from ventral progenitors and earlier than alar plate cells was based on the early thinning of the ventral progenitor area and the simultaneous appearance of differentiating motoneurons with motor axons in the ventral root before INs could be detected in the alar plate (Cajal, 1995). This was then corroborated using more modern “birth-dating” techniques (Nornes and Das, 1974; Nornes and Carry, 1978; Altman and Bayer, 2001). In addition, a ventral to dorsal gradient in the generation of spinal INs was also found. One exception is a population of dorsal commissural INs that are among the first spinal INs to start differentiating and send their axons towards the ventral commissure (Cajal, 1995; Altman and Bayer, 2001).

**Spinal cord development and neural specification**

A better knowledge of IN development is key to understand the normal and pathological development of motor circuits. However, there is very little information about how and when INs mature within the ventral horn motor circuits. More is known
about the specification of motoneurons and the regionalization of the spinal cord in different rostro-caudal and ventro-dorsal regions. This knowledge is reviewed here and could serve as guiding principles to better understand IN development.

Neural tube formation and neurogenesis and gliogenesis in the early spinal cord

Despite the variety of vertebrate nervous system organizations, the underlying principles of neural induction are maintained throughout evolution (Sanes et al. 2006). The nervous system originates from the neural plate, a region of specialized ectoderm on the upper surface of the embryo. The neural plate is already pre-patterned into forebrain, midbrain, hindbrain and spinal cord regions. This rostro-caudal specialization is reflected in the expression of different combinations of transcription factors in each of these major regions. This regionalization is imposed by morphogenetic molecules released from the underlying and paraxial mesoderm. After its induction, the neural plate undergoes a complex series of morphogenetic movements, known as neurulation, to produce a neural tube. In the spinal cord region the neural tube is located just above the notochord which is a source of important inductors for the differentiation of this region along the dorso-ventral axis.

The neural tube is initially a single layer of pseudostratified epithelium, which then proliferates rapidly. In the spinal cord, the region where the ventral folding occurred becomes the floor plate, while the dorsal region where fusion occurred during neural tube closure becomes the roof plate. Both are specialized regions that release molecules that induce dorso-ventral patterns of genetic expression in the progenitor cells located in between (Placzek, 1995; Lee et al., 2000). Later in development they
also serve to guide or repel axons, distinguishing between commissural and ipsilateral interneuronal axons (Colamarino and Tessier-Lavigne, 1995). In between the floor and roof plates, lining the ventricular zone, are the progenitor cells which are actively dividing multipotent cells (Fig.4). Waves of proliferation from progenitor cells give rise to both neurons and glia. As in other brain regions the nuclei of progenitor cells move during the cell cycle. The nuclei are close to the ventricle during mitosis and move laterally during S-phase (Sauer, 1935). Despite these nuclear movements the progenitor cells themselves do not move. Clonally related progenitors labeled in embryonic chick spinal cords with retroviruses are always found in tight dorso-ventrally restricted bands, two to three cell layers thick, within the progenitor ventricular zone (Leber and Sanes, 1995). Daughter cells that become differentiating postmitotic neurons and glia do move and migrate away from the ventricular zone and form the mantle layer while expanding laterally the thickness of the neural tube (Wentworth, 1984). The cells in the mantle layer cells undergo specific programs of differentiation and migration before acquiring their final form and connections.

The progenitor cells and early generated neurons extend initially processes that expand all the way from the ventricular to the lateral surfaces. Some progenitors become radial glial cells and keep these processes. Most cells, however, lose these processes with development. Detachment occurs because of expansion of the thickness of the spinal cord and also because neurons and glia retract these process during differentiation (Cajal, 1995; Wentworth, 1984; Fogarty et al., 2005). Most axons, with the exception of commissural axons, extend initially towards the lateral edge of the spinal cord. Motor axons exit the neural tube and extend into mesodermal layers, but
Figure 4. Anatomy of the embryonic neural tube. Top image: An E10.5 spinal cord immunostained for TuJ1 a marker of immature neurons. In this image differentiating neurons and their axons are labeled, but not the progenitor area in the middle. The outline of the spinal cord is marked with a continuous line and the border between the precursor cells in the ventricular zone and the differentiating cells in the mantle is indicated with a dashed line. Around the mantle is the marginal layer that will form the white matter and already contains a few axons at this age. DRG refers to the developing Dorsal Root Ganglia. In this particular picture it can be appreciated the axons from the DRG travelling toward the spinal cord (dorsal roots) and the exit of motor axons in the ventral roots. Ventral roots join the axons from the DRG in the periphery to form the spinal nerve. At this particular age the alar plate is rather small compared to the basal plate because the early proliferation and differentiation of motoneurons in the basal plate. Bottom diagram. Schematic of the early neural tube. At this early age is divided into the alar (dorsal) and basal (ventral) plates by the sulcus limitans. The dotted line on the left image indicates the border of the ventricular zone (VZ) which contains the progenitor cells. Differentiating cells migrate out of the ventricular zone into the mantle layer, future gray matter. The marginal layer, which develops into the white matter, contains the axons of the differentiating neurons. The spinal cord is divided into dorsal, where the sensory input enters the spinal cord and the ventral horn, where the motor output exits the spinal cord.
axons from INs become restricted to the neural tube and form the external marginal layer, which is the precursor of the white matter. Interneuron axons turn in the marginal layer and form either ascending or descending projections.

Early studies in chick and mice found that motoneurons in the basal plate differentiate first (Cajal, 1995; Wentworth, 1984). In the mouse the neural tube at lumbar regions closes around embryonic day 8 (E8). At this age the neural tube contains only ventricular progenitor cells. At E9 a few ventral neuroblasts migrate laterally and start differentiation, forming the early basal plate. These neuroblasts are motoneuron precursors and the majority of them display bipolar or unipolar morphologies and send axons away of the ventral tube through the ventral roots. By E10 many motoneurons have differentiated in the ventrolateral cord and are transitioning from bipolar to multipolar morphologies. At E11, medial and lateral subdivisions within the motor pools become obvious and there are many well-differentiated multipolar motoneurons (Fig.5, Wentworth, 1984).

The ventral proliferative regions are thinned as daughter cells become postmitotic motoneurons and leave this region to settle laterally. Proliferative zones dorsal to the motoneuron region diminish in size later. The reduction in the size of proliferative regions is always paralleled by an increase in the number of differentiating neurons in the mantle layer at the same dorso-ventral level. This observation suggested early neuroanatomists that different types of neurons arise from specific dorsal-ventral regions of the proliferative area and that different neurons are generated at different developmental times (Cajal, 1995). Different classes of neurons are therefore added sequentially in the developing spinal cord.
Figure 5. Motoneuron differentiation during very early embryonic development in the mouse. At E8 the neural tube has just closed and is composed of undifferentiated progenitors that extend processes from the ventricle to the external surface. At E9 a few postmitotic neuroblasts have emigrated from the progenitor zone and start differentiating acquiring unipolar or bipolar morphologies at the same time that one of the processes (axon) exit the spinal cord. At E10 the motoneurons display more differentiated multipolar morphologies and by E11 medial and lateral motoneurons pools start to be group together. Modified from Wentworth, 1984.
Nowadays we know that the neural tube is molecularly regionalized along the anterior-posterior and dorso-ventral axes such that each progenitor has a “molecular address code” (reviewed below) that encodes its location and defines the time window when it proliferates at the same time that restricts the types of cells that it can generate. Therefore, spinal progenitor cells are not pluripotent stem cells but partially-restricted multipotent cells from which specific subtypes of neurons and glia are generated in a spatially and temporally restricted mode. Temporal mechanisms allow the generation of different cell types over time. Cajal (1995) already suggested that spinal glial cells were generated later than neurons from the same progenitor regions. This switch is now well characterized at the molecular level for the progenitors of motoneurons and oligodendrocytes. The ventral most progenitors, named pMN and p3 respectively, give rise to motoneurons (from pMN) and a type of ventral interneuron known as V3 (from p3) during the neurogenesis phase. Later these progenitors give rise to oligodendrocytes (Soula et al., 2001; Richardson et al., 1997; Zhou et al., 2001; Lu et al., 2000). This temporal switch in specification to oligodendrocytes is accompanied by a change in the pattern of transcription factor expression in progenitors, which results in part from changing levels of sonic hedgehog (Shh) expressed by the notochord and floor plate during development. Olig2, a basic helix-loop-helix (bHLH) transcription factor, is expressed in a restricted domain of the spinal cord ventricular zone that sequentially generates motoneurons and oligodendrocytes. Just prior to oligodendrocyte generation, the domains of Olig2 and Nkx2.2 expression switch from being mutually exclusive to overlapping and the proneural Neurogenins 1 and 2 are extinguished within this region promoting glial differentiation. Coexpression of Olig2 with Nkx2.2 in the spinal cord
promotes oligodendrocyte differentiation. Olig2 therefore functions sequentially first in motoneuron generation and then in oligodendrocyte fate specification. This dual action is enabled by spatio-temporal changes in the expression of other transcription factors with which Olig2 functionally interacts.

In conclusion, a major contributor to define cell fate in the embryonic spinal cord is the position of its progenitors, being the type of cell derived from each progenitor also regulated and influenced by the time of generation. Progenitor mitotic activity peaks at different times in a ventro-dorsal sequence within the ventricular proliferation area and these results in a parallel ventral to dorsal progression of neurogenesis and differentiation. In this thesis we will ask whether time of generation, from a single progenitor zone, is also important to define different neural phenotypes within subgroups of ventral INs.

**Time of neurogenesis and neuronal type specification**

Timing of neurogenesis is an important factor in generating neuronal diversity in other regions of the central nervous system. For example, different types of retinal cells arise from the same progenitors in a temporally regulated manner (Cepko et al., 1996). Similarly, it is well known that time of neurogenesis defines laminar location and axonal projections of pyramidal cells in the cortex (Rakic, 2009). As will be reviewed later, the time of “birth” is also correlated with columnar identity in motoneurons. Little, however, is known about the importance of time of birth for generating diversity within interneuronal groups. Different types of cortical INs arise from different proliferative domains in the ganglionic eminences, but in addition a
single region can give rise to multiple subtypes (Anderson et al., 1997; Wonders and Anderson, 2006). For example, cortical INs classified as fast-spiking (parvalbumin-expressing basket and chandelier INs) and the regular burst-spiking somatostatin expressing cells all arise from progenitors in the median eminence. Interestingly, the period of neurogenesis of parvalbumin vs. somatostatin cells is different (Butt et al., 2007). Within the spinal cord recent analyses in zebrafish suggested that within a single class of excitatory ipsilateral INs sending descending connections to motoneurons, cells generated at different times become located in different dorso-ventral regions, connect with different classes of motoneurons and are differentially recruited depending on the speed of swimming (Kimura et al., 2006; McLean and Fetcho, 2009). Our studies will try to determine if time of generation is not only capable of imposing functional and connectivity gradients within a single class of INs, but whether it can also generate completely different types of adult INs.

**Molecular mechanisms of cell specification along the dorso-ventral axis**

Cellular specification along the dorso-ventral axis occurs by inductive mechanisms that regulate expression of certain transcription factors that control the fate and differentiation of neurons. These mechanisms are mediated by secreted factors emanating from the ventral notochord and floor plate, and dorsally from the non-neural ectoderm (Jessell, 2000; Poh et al., 2002; Melton et al., 2004). Diffusion of secreted factors from these sources results in concentration gradients along the dorso-ventral axis that are converted from graded signals into all-or-none distinctions in cell fate (Fig.6, Briscoe et al., 2000). In addition, these gradients change with development and
perhaps influence the temporal sequence of neuronal generation from each dorso-ventral domain, although less is known about this possibility.

The differentiation of ventral cell types is triggered by signals from a gradient of the protein Sonic hedgehog (Shh), secreted initially by the notochord and later by floor plate cells (Placzek, 1995; Chiang et al., 1996). In addition, retinoids derived from the paraxial mesoderm and possibly also from differentiating neural cells, provides a parallel signaling pathway that aids in the specification of some IN subtypes (Zhao et al., 1996; Sockanathan and Jessell, 1998, Pierani et al., 1999). Sonic hedgehog and retinoids control the expression of different transcription factor combinations in progenitors located at different dorso-ventral regions (Fig. 6).

Briscoe et al. (2000) proposed that the ventral progenitor domains emerge in three main stages. In the first stage, homeodomain proteins expressed by ventral progenitors interpret the graded Shh signaling. This Shh gradient represses expression of class I transcription factors (Pax7, Pax6, Irx3, Dbx1, and Dbx2) and induces class II factors (Nkx6.1, and Nkx2.2) with each protein induced or repressed at different levels by different Shh concentrations. As a result, a dorso-ventral gradient for the expression for each transcription factor is established. In the second stage, the selective reciprocal repression between class I and II proteins refines the progenitor domain boundaries (reviewed in Price and Briscoe, 2004). Such repressive interactions occur at certain expression thresholds and are all-or-none, that is one transcription factor in the pair is completely repressed. They have three roles: First, they define the dorsoventral limits of expression of class I and class II proteins. Second, they ensure the existence of sharp boundaries between progenitor domains. Third, they relieve progenitor cells of a
Figure 6. Scheme of ventral progenitor domains giving rise to ventral interneurons and motoneurons. Each ventral canonical class of interneuron is characterized by the expression of a characteristic transcription factor (indicated) and originates in one specific progenitor domain. These differences in progenitor domains are induced by dorso-ventral concentration gradients of the proteins Shh and BMP as indicated in the top right corner. Shh is released from notochord (N) and the floor plate (FP). BMPs are released from the roof plate (RF).
V1 generation

Class/transcription factor

Progenitors

- p0
- p1
- p2
- pMN
- p3

- V0 \rightarrow Evx1
- V1 \rightarrow En1
- V2 \rightarrow Chx10 and GATA3
- MN \rightarrow HB9
- V3 \rightarrow Sim1

BMP
Shh
requirement for ongoing Shh signaling, consolidating progenitor domain identity. In the third stage, the homeodomain protein code is translated into specific neuronal subtype identity. As a consequence the combinatorial expression profile of these proteins leads to five progenitor domains in the ventral neural tube. These are defined as pMN, which gives rise to motoneurons and p0 to p3 giving rise to four classes of embryonic ventral INs (Fig.6, Briscoe et al., 2000; Jessell, 2000; Poh et al., 2002; Goulding and Lamar, 2000).

For Shh-induced dorso-ventral progenitor domains to remain stable, it is necessary to constrain cell movements within the ventricular zone as shown by the retroviral experiments of Leber and Sanes (1995). Once these boundaries are established, progenitor cells freely move within a given domain but are unable to cross into adjacent domains, thereby establishing lineage-restricted compartments in the ventral neural tube (Goulding and Lamar, 2000).

**Anterior-posterior patterning**

Similar induction and repression mechanisms of transcription factor expression help to define the rostro-caudal organization of the spinal cord. The rostrocaudal pattern is in part imposed by retinoic acid (RA) and fibroblast growth factor (FGF) signals. It has also been described that early Wnt (Wingless Int, Wingless is a recessive mutation affecting wing development in *Drosophila melanogaster* and Int is an homologus gene of Wingless with common evolutionary origin) signaling provides a positional context for the later actions of RA and FGF in specifying rostrocaudal regional identity in the embryonic spinal cord (Nordström, et al., 2006; Dasen and Jessell, 2009). The
differential expression of FGFs, RA and Wnts control the expression of the Hox gene family, a class of transcription factors with an evolutionarily conserved role in establishing differences in cell identity along the rostrocaudal axis (Melton et al., 2004). Hox genes are localized in gene clusters and their position in the cluster defines its expression pattern; genes located at the 3’ end of the cluster are expressed more anteriorly in the neural tube than genes at the 5’ (Melton et al., 2004). Hox gene expression in the spinal cord is closely aligned with their position within the Hox cluster and this is further refined by cross-repressing mechanisms similar to those between class I and class II proteins involve in dorso-ventral patterning (Dasen et al., 2009). Hox genes are therefore informative markers of the rostrocaudal positional identity of progenitor cells and are also determinants of motoneuron identity, both in the hindbrain (cranial nerve motor nuclei) and spinal cord. For example, Hox9 expression in progenitors and cross-repressive interactions between Hox6 and Hox9 proteins in postmitotic motoneurons consolidate the distinct profile of the lateral motor column (LMC) in the cervical enlargement and the Column of Terni (CT, i.e. preganglionic sympathetic neurons) in the thoracic spinal cord. Hox6 activity in brachial motoneurons directs RALDH2 (retinaldehyde dehydrogenase-2) expression and induces late features of LMC identity, while Hox9 activity in thoracic motoneurons directs BMP5 expression and the dorsal migration of “visceral” motoneurons (Dasen et al., 2003). It is not known yet if similar mechanisms act on rostro-caudal specification of IN identity.
The “V code”

Dorso-ventral patterning results in 5 ventral progenitor domains that give rise to motoneurons and 4 types of INs, known as V0, V1, V2, and V3. Postmitotic neurons generated from each domain upregulate specific transcription factors that then define the class. V0 cells are characterized by expression of Evx1/2 (Even-skipped homeobox1), V1 cells by engrailed-1 expression (En1), V2 cells are divided in cells that express GATA3 (V2b INs) or the CEH10 Homeodomain-Containing Homolog (Chx10; V2a INs) and V3 cells express Sim1 (Single-minded homolog1). These cell-type specific genes have been extensively used to direct the expression of reporter genes like green fluorescent protein (GFP), yellow fluorescent protein (YFP), variants of red fluorescent proteins, like tdTomato, and LacZ to specific subclasses of ventral INs. They have also been used to direct genetic silencing or deletion of each of these populations either in embryo or in the adult. Each type is considered a canonical cell type with some basic common properties. Taking advantage of genetic labeling it was found that each embryonic subgroup is characterized by a different migration and final location, the direction of extension of the primary axon and frequently (but not always) its neurotransmitter phenotype. These properties are conserved through evolution and each cell type shares similar fundamental properties from fishes to mammals (Goulding, 2009; Grillner and Jessell, 2009).

V0 INs take a ventro-medial migration and settle in the future LVIII extending commissural projections that distribute for 1-4 segments rostrally in the contralateral spinal cord. Most V0 INs display inhibitory phenotypes, but some are also excitatory (Lanuza et al., 2004). These authors also used an in vitro spinal cord preparation to
induce fictive locomotion by application of N-methyl-D-aspartic acid (NMDA) and 5-hydroxytryptamine (5-HT, serotonin) and recorded alternating bursts of motor activity in left lumbar 2 (IL2) and right lumbar 2 (rL2) ventral roots. After genetic deletion of V0 INs there was a loss of strict left-right alternance in motor output and sometimes ipsi and contralateral roots fired synchronously. The authors concluded that V0’s are involved in stabilization of the left-right alternation of motor output. Recently, a subclass of V0 INs (V0c) was defined according to the expression of the pitx2 transcription factor. V0c neurons are cholinergic and are located close to lamina X. These cells are the origin of C-terminal synaptic boutons on motoneurons. Genetic deletion of this group showed they control motoneuron excitability (Zagoraïou et al., 2009).

V1 INs in contrast, migrate latero-ventrally and most end up located in LVII in close apposition to the lateral motor pools (Matise and Joyner, 1997; Saueressig et al., 1999; Alvarez et al., 2005). They project ascending axons that travel for short distances in the ipsilateral ventro-lateral funiculus and project to ipsilateral motoneurons. So far, only inhibitory phenotypes have been found in this subclass (Saueressig et al., 1999; Sapir et al., 2004; Alvarez et al., 2005). V1 INs are therefore good candidates to provide local inhibitory modulation to motoneurons. Both, Renshaw cells and IaINs have been shown to derive from this group (Sapir et al., 2004; Alvarez et al., 2005). Genetic silencing of V1 INs in embryo or postnatally did not affect left-right or flexor-extensor coordination during NMDA/5-HT induced fictive locomotion in the in vitro spinal cord preparation, but the step cycle duration was lengthened leading to the conclusion that V1 INs regulate the speed of locomotion (Gosgnach et al., 2006). Later
it was shown that reciprocal inhibition between quadriceps and biceps is not altered in Pax6 knockout mutants in which V1 INs are not generated (Wang et al., 2008). Taken together with the lack of effect of V1 deletions in fictive locomotion flexor-extensor motor output alternance (Gosnach et al., 2006) and with more recent data describing IaINs in the mature spinal cord that are not derived from V1 INs (Siembab et al., 2010) the results suggest multiple origins for IaINs. In this thesis we will refer to the cells under study as “V1-derived” IaINs.

V2 INs also follow a lateral migration but do not extend as ventrally as V1’s, they also project axons to the ventro-lateral funiculus, but these are mostly descending axons (Lunfald et al., 2007; Al-Mosawie et al., 2007). Most V2 INs express Chx10 and are excitatory (V2a subtype), however a smaller subgroup expresses GATA3 and these are inhibitory (V2b subtype). Ablation of V2a INs using transgenic Chx10-DTA mice (which express diphtheria toxin specifically in Chx10 expressing neurons) lead to deficits in left-right coordination, similar to the observation after deletion of V0 INs, in the in vitro fictive locomotion spinal preparation (Crone et al., 2008). These authors then used genetic labeling to demonstrate a direct connection between Chx10 on V0 neurons. Interestingly, in the whole animal these deficits are more apparent at high treadmill speeds and the mice switch from normal alternating running gaits to an abnormal synchronous rabbit-like pattern (Crone et al., 2009). These results suggest that similar to homologous zebrafish V2a (Alx) INs, mammalian V2a INs are functionally diverse and their overall functional impact might change with locomotor speed. Correspondingly, recent studies on the cellular properties of V2a cells demonstrated a variety of morphologies, axonal projections, electrophysiological
properties and coupling of their firing activity with rhythmic motor outputs (Zhong et al., 2010; Dougherty and Kiehn, 2010).

Lastly, V3 INs are generated most ventrally and take a medio-dorsal migration pathway that divides them into three different subgroups settling in different dorso-ventral regions. V3’s are excitatory and extend contralateral axons and therefore they might be important in synchronizing motor activity between both sides of the spinal cord (Zhang et al., 2008). Blocking neurotransmission from V3 INs using conditional expression of Tetanus neurotoxin in these cells increased the duration of the step cycle and made motor bursts more variable and labile. The authors concluded that V3 INs stabilize locomotor network rhythmicity (Zhang et al., 2008). In a recent study of netrin knockout mutants it was found that all V0 commissural INs (mostly inhibitory) fail to cross axons to the other side of the spinal cord, however V3 axons (excitatory) were netrin-independent and crossed normally (Rabe et al., 2009). These animals express a necessary rabbit-like hopping gait with synchronous activation of left and right motoneurons.

Although these experiments point to possible functions of the different classes of embryonic INs, they are difficult to interpret because they are broad deletions that do not take into account the diversity of functional subtypes within each class. Moreover, none of the specific cell deletions seems able to eliminate locomotion rhythmicity. The possibility of compensatory and redundant mechanisms cannot be overlooked but in addition we need a better understanding of the variety of IN subclasses derived from each class and their mechanism of differentiation to assess more precisely their function. An important example is the V0c group which controls the excitability of
ipsilateral motoneurons and therefore has a function quite different from most cells in
the generic V0 group (Zagoraiou et al., 2009).

Further refinement of approaches used to classify ventral INs will likely become
highly valuable as demonstrated in animals with simpler spinal circuits, such as the
zebrafish (Fetcho and Bhatt, 2004). In these animals there is a nice correlation between
canonical subtypes and one or a few types of well-defined INs in the mature spinal
cord, each of known morphology, neurotransmitter phenotype and functional action.
However, in mammals there are many more classes of ventral INs in the adult spinal
cord than canonical embryonic subtypes. It has been argued that this is the result of
evolutionary pressures towards greater diversification to allow the transition from
swimming to terrestrial locomotion and encode the more complex and larger number of
motor patterns displayed by terrestrial mammalian species (Alvarez et al., 2005;
Goulding and Pfaff, 2005; Goulding, 2009; Grillner and Jessell, 2010).

Although the mechanisms that lead to the diversification of the generic V0-V3
INs have not been defined, there is an interesting parallel in the specification of
motoneurons into classes (somatic vs. visceral), columns (lateral vs. medial) and
specific pools (reviewed in Jessell, 2000; Price and Briscoe, 2004). This diversification
of motoneuron phenotypes is characterized by the sequential expression of distinct
transcription factors driven by a combination of intrinsic and extrinsic signals. It is
possible that similar hierarchical specialization also occurs during IN development.
Mechanisms of motoneuron diversification

The process by which motoneurons develop unique identities depends on a hierarchical and sequential expression of transcription factors that increasingly restrict motoneuron differentiation based on cell body position, axonal projections and gene expression. The specification of generic motoneuron identity leads into the generation of motoneuron subtypes located at specific positions in the spinal cord. The soma of functionally related groups of motoneurons that are destined to share common projection targets settle in longitudinally oriented columns as their axons project towards their target regions. Finally, motoneurons that innervate the same muscle form clusters known as motor pools and at this time is when pre- and post-synaptic connections are made. Each step involves extracellular signals that regulate intrinsic cell-autonomous determinants of motor identity (reviewed in Price and Briscoe, 2004).

Generic motoneuron identity is specified by the combinatorial action of three homeodomain proteins, Nkx2.2, Nkx6.1, and Irx3 expressed in three adjacent ventro-dorsal progenitor domains induced by the graded action of Shh. Ventrally Nkx2.2 (in the p3 domain) and dorsally Irx3 (in the p2 domain) repress motoneuron differentiation and ensure that motoneuron generation is restricted to the pMN domain expressing Nkx6.1. Within pMN, Nkx6.1 induces transcription factors that are essential for motoneuron specification, such as Olig2 and MN2 (the chick homolog of mammalian Hb9). Initially, Olig2, a bHLH protein, induces proneural genes like neurogenin 2 (Ngn2) and favors the acquisition of motoneuron properties by repressing Irx3. Later, when Nkx2.2 expression moves dorsally into the pMN domain, Olig2 represses proneural genes and oligodendrocytes are generated (reviewed before, pag. 27). In the
absence of Nkx2.2, Olig2 induces MNR2 (chick) and Hb9 (mammals) in the progenitors during their final cell division, and these determine motoneuron identity.

During early differentiation, motoneurons are also subdivided into subclasses that innervate different muscles in the periphery. After leaving the spinal cord, motor axons project either dorsally, towards axial muscles or ventrally towards body wall muscles or limb muscles. Motoneurons innervating these distinct regions become positioned into longitudinal columns. Motoneurons located medially in a subcolumn called MMCm innervate axial muscles. This column is present throughout the rostro-caudal extent of the spinal cord. More laterally in the spinal cord are the subcolumns that project to body wall muscles (MMCl) and to limb muscles (LMC). LMC motoneurons are present only at cervical and lumbar levels while MMCl motoneurons are present only at thoracic levels. LMC axons face a second choice at the base of the limb where they project to dorsal or ventral limb muscles. The lateral LMC subcolumn projects to dorsally derived muscles and the medial LMC subcolumn projects to ventrally derived muscles. Each of these subcolumns can be identified by the combinatorial expression of LIM homeodomain transcription factors, like Isl1, Isl2, Lim1, and Lim3, prior to the innervation of muscle (reviewed in Price and Briscoe, 2004). The formation of subcolumns within the LMC is related to time of birth and an inside-out migration that has a critical influence on their identity. Isl1 and Lim1 distinguish medial from lateral LMC motoneurons. Isl1 is initially expressed by all LMC neurons just after they are generated but then is rapidly downregulated from lateral LMC neurons at which time Lim1 is induced. The switch in LMC subtype depends on retinoid signals provided by earlier-born LMC motoneurons. Early born
LMC motoneurons form a medially located column and later born LMC motoneurons pass through them to reach more lateral positions. LMC neurons upregulate the retinoid synthesizing enzyme RALDH2 and exposure of later-born naïve LMC neurons to retinoids represses Isl1 and promotes Lim1 expression (Sockanathan and Jessell, 1998).

Later, motoneurons differentiate into pools dependent on expression patterns of a different family of transcription factors named E-twenty six or ETS (Lin et al., 1998). The onset of ETS gene expression occurs at late developmental stages and coincides with limb innervation. Recently, many other genes related to specific adhesion and recognition mechanisms, like ephrins, cadherins, semaphorins, have been found expressed in specific motor pools, sometimes under the control of specific transcription factor combinations (reviewed in Price and Briscoe, 2004).

In summary, motoneuron diversification suggest progressive acquisition of properties from the more general column specification to specific motor pool identity influenced by extrinsic signals that trigger intrinsic programs of differentiation. It is possible that similar principles can be transferred to IN diversification.

**V1-derived interneurons in the mature spinal cord: basic control of motoneurons**

As mentioned before, inactivation or deletion of V1 INs results in a marked prolongation of the step cycle and slows the motor rhythm, suggesting they are crucial for setting the speed of locomotion (Gosgnach et al., 2006). Core features of V1-INs, like their inhibitory nature and ipsilateral projections, are conserved between the aquatic vertebrates and mammals. The seemingly evolutionary conserved role of V1 neurons in regulating the speed of the locomotor rhythm suggests that certain functions
may have been preserved between the swimming and walking CPG and reflects the close phylogenetic relationship between spinal neurons in swimming vertebrates and their terrestrial counterparts. This is particularly apparent in the embryonic spinal cord. Although it was initially suggested that V1-INs form a homogenous population of inhibitory interneurons in mice embryos (Sauressig et al., 1999), it was quickly shown that at least in the chick embryo they express heterogenous electrophysiological properties (Wenner et al. 2000). However, in “simpler” vertebrates (fish and tadpoles) V1 INs remain a homogenous population of ipsilaterally projecting, glycinergic inhibitory INs that exert motor control by limiting firing of motoneuron and INs and gating sensory information during swimming (Higashijima et al., 2004; Li et al., 2004). Interestingly, these two functions are provided in the mammalian spinal cord by two different classes of adult INs, Renshaw cells and IaINs, and both were found to be derived from embryonic V1 INs in the mouse (Sapir et al., 2004; Alvarez et al., 2005). V1 INs in the mammalian embryonic spinal cord therefore represent a primitive ground state that undergoes diversification during development and gives rise to specialized INs with more restricted functions.

**Renshaw cells: recurrent inhibition**

*Physiology and function*

Renshaw cells mediate recurrent inhibition (Fig.7). Recurrent inhibition was the first inhibitory spinal pathway identified due to the simplicity of its organization and its unique feature of being activated by motor axons. In 1941, Renshaw discovered that in animals with dorsal roots sectioned, antidromic impulses in motor axons decrease the
excitability of α-motoneurons projecting to the same or synergistic muscles. Renshaw named this effect recurrent inhibition and in 1946 identified a group of INs that he proposed mediated the inhibitory effects of motor axon activation on motoneurons. A few years later Eccles and colleagues (1954) discovered that the spinal recurrent inhibitory pathway was disynaptic, involving a cholinergic synapse from motor axon collaterals onto an IN located in the ventromedial portion of the ventral horn and a strychnine-sensitive hyperpolarizing synapse from this IN onto the motoneurons. He named this IN the Renshaw cell. Later it was found that Renshaw cells also control IaINs and therefore reciprocal inhibition and the amount of co-contraction between antagonist muscles (Hultborn et al., 1971) (see schematic in Fig.7). Renshaw cells and recurrent inhibition are thought to play a variety of different roles in fine-tuning motor output by modulating motoneuron recruitment and proprioceptive reflex circuits (reviewed in Windhorst, 1996).

Location, anatomy and morphological identification

The ‘Renshaw cell area’ was identified in ventral lamina VII (Thomas and Wilson, 1965). The location, morphology and glycinergic/GABAergic nature of Renshaw cells was confirmed by combining intracellular recording and labeling with immunolabeling (Fyffe, 1990; 1991a,b). Renshaw cells are small size INs located in the exit region of motor axons in ventral lamina VII and IX and extend relatively small dendritic arbors. Their axons extend rostro-caudally through a few ipsilateral spinal segments and make local arborizations preferentially in lamina IX.
Later it was found that Renshaw cells uniquely display a high density of proximal inhibitory synapses with uncommonly large postsynaptic densities that can be labeled by gephyrin, a glycine receptor and GABA<sub>A</sub> receptor clustering protein (Alvarez et al., 1997). Using this criteria for identification Carr and colleagues (Carr et al., 1998) confirmed an earlier suggestion that primate Renshaw cells express high levels of the calcium-buffering protein calbindin (Ardvisson et al., 1992). Developing Renshaw cells can also be distinguished as a distinct cluster of ventrally located calbindin-IR cells in neonates (Geiman et al., 2000) and embryos (Sapir et al., 2004). Moreover, it appears that many other ventral spinal INs express calbindin initially, but then it is downregulated during postnatal development in most, except in the Renshaw cells (Zhang et al., 1990; Siembab et al., 2010). Thus, calbindin expression appears to be an intrinsic feature of the Renshaw cell phenotype but it’s function and the difference in the regulation of its expression in Renshaw cells compared to other INs have not been studied.

**Ia inhibitory interneurons (IaINs): reciprocal inhibition**

*Physiology and function*

In 1897, Sherrington demonstrated that the contraction of a muscle is accompanied by the relaxation of its antagonist and denominated this effect “reciprocal inhibition”. Lloyd (1941) postulated that reciprocal inhibition was mediated by Ia afferents directly affecting motoneurons, however, more than a decade later, Eccles demonstrated in 1956 that an IN was interpolated in the reciprocal inhibitory pathway.
This interneuron was called “Ia intermediate interneuron”, which evolved into its current name, “Ia inhibitory interneuron” (Fig.8).

The reciprocal inhibitory circuit as we know it today involves Ia afferents originated in the muscle spindle primary endings that exert monosynaptic excitatory action onto homonymous motoneurons and activate IaINs inhibiting the motoneurons of the antagonistic muscle. IaINs have been also shown to inhibit other IaINs (Hultborn et al., 1976) and to be modulated by Renshaw cells (Hultborn et al., 1971).

Location, anatomy and morphological identification

IaINs ventral horn position was determined first from electrophysiological recordings (Hultborn et al., 1971). More specific information on exact location came from intracellular labelings of IaINs. They were found in lamina VII (LVII) dorsal or medial to lamina IX (Burke et al., 1971). IaINs are always located in the same spinal cord segment as the Ia afferents that excite them and therefore to exert reciprocal inhibition between pools of motoneurons located segments away the axons of IaINs need to travel into the lateral and ventral funiculi where they ascend or descend sending collaterals to motor pools locally or several segments away (Jankowska and Lindstron, 1972). A thorough investigation of IaIN locations, morphologies, dendritic arbors and axon trajectories demonstrated considerable variability within this population (Rastad et al., 1990). IaINs were located in all regions of lamina VII, could exhibit large or small cell bodies and dendritic arbors and their axons could be preferentially descending or ascending or bifurcating, some being quite local and others long range propriospinal.

Histological identification of IaINs is more complicated than for Renshaw cells, since synaptic connectivity needs to be identified and IaINs seem very heterogeneous.
A few years ago our lab found that some INs in the ventral horn receive a dense innervation in the cell body and proximal dendrites from Renshaw cells (labeled with calbindin antibodies) and Ia afferents (labeled with the proprioceptive synaptic marker VGLUT1; Todd et al., 2003; Alvarez et al., 2004). In parallel with electrophysiological criteria (see Alvarez and Fyffe, 2007) these INs were identified as IaINs (Alvarez et al. 2005; Siembab et al., 2010). We don’t know the proportion of IaINs identified using this criteria or whether cells with such high density of Renshaw cell and Ia afferent inputs represent a subpopulation of IaINs. These criteria, however permit the identification of some cells that with very high probability are IaINs. Up to date there are no other histological or genetic markers to label this population. Developmental analyses will hopefully aid in the discovery of specific genes more specifically expressed in IaINs and perhaps these will subdivide the IaIN population in to further subgroups each having more homogenous morphological and functional properties.

The basic question pursued in this thesis is when and by what possible mechanisms Renshaw cells and IaINs differentiate from each other within the V1 population. The results should be always interpreted considering that it is unlikely we identified all V1-derived IaINs throughout our studies. The IaIN population we can identify, however, might give important information on the mechanisms of differentiation and in comparison to Renshaw cells. Based on previous analyses we believe that recognition of the Renshaw cell population is quite complete (Alvarez and Fyffe, 2007). The starting point for generating our hypothesis is the observation that both cell types can be recognized in the neonatal spinal cord (Siembab et al., 2010). This information together with studies in human newborns and mice pups, showing that
recurrent and reciprocal inhibition are functional at birth (Mc Donough et al., 2001; Wang et al., 2008) strongly suggest that the main cellular elements and connections are preformed and specified in the embryo. Up-to-date there is no information about the mechanisms that specify any of the INs found in the adult spinal cord and classically defined according to their function (as reviewed in Jankowska et al., 1992).
Figure 7. Diagram of basic connections between IaINs, motoneurons, Ia afferents and Renshaw cells.
Figure 8. Diagram of the basic connections of reciprocal inhibition through IaINs. IaINs receive inputs from Ia afferents that innervate muscle antagonists of the motor pools they inhibit. Reciprocal inhibition is extensive for excitatory inputs from other sources like descending inputs. Descending pathways are represented in a discontinued line. Descending pathways co-activate α-MNs and corresponding IaINs. In general, IaINs receive the same excitatory input than the motor pools receiving input from common Ia afferents. In this way reciprocal inhibition is not limited to the Ia afferent mediated stretch reflex but also to all excitatory inputs allowing reciprocal excitation-inhibition of flexors and extensors. In addition, extensor-coupled IaINs inhibit antagonist flexor-coupled IaINs, and vice versa. Further modulation of the IaIN is through Renshaw cell inputs.
III. HYPOTHESIS AND SPECIFIC AIMS

Hypothesis: Differentiation of V1-INs into Renshaw cells and IaINs occurs early in mouse embryos and depends on birth-date, early expression of specific transcription factors and different spatial relationships during early migration.

Aim 1: Determination of birthdates of different populations of V1-derived INs.

Hypothesis 1: Renshaw cells and V1-derived IaINs differ in their time of generation from p1 progenitors.

Previous studies suggested that V1 INs exit the progenitor zone between E9 and E12 (Matise and Joyner, 1997; Saueressig, et al., 1999). We tested the possibility that different classes of V1-INs have different birthdates within this period by pulse-labeling with bromodeoxyuridine (BrdU) newly generated cells at different embryonic times. Embryos from animals encoding reporters for V1-IN identification, either LacZ (En1Cre/Tau-LacZ) or YFP (En1Cre/Thy1-YFP), were injected with BrdU at embryonic ages E9.5 to E12.5. BrdU incorporation into Renshaw cells and V1-derived IaINs was analyzed postnatally at P15, an age in which each cell type has differentiated most of their characteristics (Siembab et al., 2010).

Aim 2: Characterization of transcription factor expression of Renshaw cells and IaINs in the embryonic and postnatal spinal cord.

Hypothesis 2: Renshaw cells and V1-derived IaINs start their differentiation immediately after being generated by expressing cell-type specific transcription factors.
In a previous preliminary study it was suggested that subpopulations of V1 INs could be differentiated based on the expression of the transcription factors FoxP2 and MafB (Geiman et al., 2007). We tested in this aim whether these transcription factors are specifically expressed in Renshaw cells and V1-derived IaINs. Because these transcription factors are quickly downregulated after birth we analyzed using immunohistochemistry their expression in Renshaw cells and IaINs at P0 and P5. Then we analyzed the upregulation of their expression in early embryos. To obtain early expression in embryo of V1 genetically encoded reporters we used a new reporter mouse (En1-Cre/Rosa26-tdTomato).

Aim 3: Characterization of the migratory pathway of Renshaw cells as distinct to that of other V1-INs.

Hypothesis 3: Early Renshaw cells follow a unique migratory path that influences their unique relationship with motor axons.

The analyses in embryonic spinal cord performed in Aim2 suggested calbindin is an early marker of newly born Renshaw cells and that specific transcription factors in these cells are expressed only after they have settled in their final positions. We therefore hypothesized that the migration pathway has implications for Renshaw cell differentiation and could explain their unique relationship with motor axons. To analyze migrating Renshaw cells in relation to motoneurons and ventral root axons we labeled motoneurons with the transcription factor islet1 and ventral roots with a monoclonal antibody against a class III beta-tubulin isoform (Tuj1) characteristic of immature neurons and axons.
IV. GENERAL METHODS

Animal models for V1-interneuron identification

Three animal models were used in this study to identify V1-interneurons (V1-INs) in embryonic or postnatal tissue sections. A Cre/lox recombination strategy was used to direct expression of reporter genes (LacZ, YFP, and tdTomato) in cells derived from engrailed-1 expressing V1-INs (Sapir et al., 2004). All animals were obtained by crossing En1^{Cre+} heterozygotes (Sapir et al., 2004) with three different reporter mouse lines. All reporter lines contain a transcriptional stop cassette flanked by two loxp sites just upstream of the reporter gene. In the driver line, the cre recombinase gene was inserted into the first coding exon of engrafted-1 (enl), a transcription factor expressed by V1-INs during development. In this manner only V1-INs express Cre in the spinal cord. Cre is a type I topoisomerase from P1 bacteriophage, that catalizes site-specific recombination of DNA between loxp sites. Cre recombination of loxp sites removes the stop signal and allows transcription to proceed onto the reporter gene (Fig.9). Expression is continuous through the life of the V1-derived neuron due to the activity of the promoters that control the reporter genes expression. The three reporter lines we used are described below.

- Tau-lox-STOP-lox-mEGFP-IRES-NLS-LacZ:

  Tau-lox-STOP-lox-mEGFP-IRES-NLS-LacZ mice (Tau-LacZ, Hippenmeyer et al., 2005) contain one copy of the reporter transgene knocked-in by targeted recombination in the tau gene. The Tau promoter is reportedly active in embryonic and
adult neurons (although we noted when using this line that is not active in some neurons at the earliest embryonic ages: see results Aim 2). The reporter gene contains a bicistronic element with an internal ribosomal entry site (IRES) that allows expression of multiple proteins from a single mRNA transcript as ribosomes bind to the IRES in a 5'-cap-independent manner to initiate translation. The bicistronic element is a single transcript with open reading frames encoding for two different proteins separated by the IRES sequence. The first coding sequence generates a modified myristoylated EGFP (mEGFP) designed to bind to the plasma membrane and label entire cell surfaces. However, in our tissue sections it only labels the axons. The second coding sequence (NLS-LacZ) translates a modified bacterial β-galactosidase with a nuclear signal, meaning the localization of the reporter will be restricted to the nuclei of the cells. We used immunodetection of NLS-LacZ to identify the location of V1 cells (Fig.10). mEGFP remained undetected in our experiments and since immunocytochemical amplification is necessary for revealing axonal mEGFP in these animals, we were able to reserve the green channel for other immunomarkers.

- Thy1-lox-STOP-lox-EYFP:

    The Thy1-lox-STOP-lox-EYFP mouse line (Thy1-YFP, Feng et al., 2000; Buffelli et al., 2003) contains multiple copies of a transgene that will produce Enhanced Yellow Fluorescent Protein (EYFP) after Cre/lox recombination. EYFP is a yellow-shifted Green Fluorescent Protein (GFP). In this line YFP expression fills the cell bodies, dendritic arbors and axons of V1-derived INs (Fig.11). The Thy1 promoter is robustly expressed in postnatal neurons (but not in embryo), however one significant
property of Thy1 lines is expression “mosaicism” (Feng et al., 2000). This refers to the characteristic of Thy1 reporters to become active in only a percentage of neurons in the target population. We chose the Thy1 line 15 because it labels a large percentage (~75%) of all V1-derived INs (Siembab et al., 2010). Complete cellular filling, as provided by YFP expression in this line, is advantageous in experiments trying to identify V1-derived IaINs. This is because our criteria are based on synaptic contacts and good definition of the cell body and dendrites is necessary for this.

- CAG-Rosa26-lox-STOP-loxp-tdTomato-WPRE:

These mice harbor a targeted mutation of the Gt(Rosa)26Sor locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato) that is expressed only after Cre recombination. tdTomato is a modification of the Red Fluorescent Protein (RFP) of marine invertebrate organisms such as the soft coral and reef coral (Madisen et al., 2010). The wild type RFP protein, which is an obligate tetramer, is not well tolerated in mammalian systems. The original molecule was modified to optimize expression in mammalian cells. The modified tdTomato is among the brightest fluorescent proteins available. The CAG promoter is a combination of the cytomegalovirus and chicken beta-actin promoter and induces high gene expression in mammalian cells while the Rosa26 locus is a site that permits reliable and efficient expression of transgenes targeted to that locus. As a result tdTomato expression in these animals is strong and was always visualized “naked” in our studies using epifluorescence or confocal microscopy (Fig. 12).
Figure 9. Conditional cre/lox recombination used to label V1-interneurons. The reporter lines contain a reporter cassette in which a loxp-STOP-loxp sequence is upstream of the reporter gene. This stop signal prevents transcription of the reporter in most cells. Loxp sites recombine in the presence of Cre, such that the DNA fragment in between (in this case a transcriptional STOP signal) is deleted. Cre is expressed specifically in *engrailed-1* expressing V1 neurons and therefore reporter expression is only allowed in these cells.
Figure 10. Labeling in En1-Cre / Tau-LacZ mice. Low magnification images of a P15 spinal cord hemisection from the En1-Cre / Tau-LacZ animal. The sections were triple immunolabeled for LacZ (β-galactosidase, 405, blue), calbindin-immunoreactivity (CB-IR, FITC, green) and parvalbumin-immunoreactivity (PV-IR, Cy5, white). A) LacZ-IR labels the nuclei of V1-derived INs. V1-derived INs are distributed through the ventral horn of the spinal cord mostly concentrated medial to lumbar motor pools. B) Calbindin-IR distinguishes a group of ventrally located neurons that correspond with Renshaw cells (RC area). In addition, a few other ventral calbindin-IR cells are located more dorsally. These are more frequent in upper lumbar regions and were divided into large (see big CB) and small cells according to soma size. Many other calbindin-IR cells are located in the dorsal horn. Dorsal horn calbindin-IR cells do no belong to the V1-derived population. C) Parvalbumin-IR is present in the axons of proprioceptive afferents and in many dorsal and ventral horn INs. By difference to CB, Parvalbumin-IR cells are more varied being distributed in all dorso-ventral and medio-lateral regions of the ventral horn, including a proportion of the RCs. D) Calbindin-IR and LacZ staining shows Renshaw cells are labeled with LacZ confirming they are V1-derived interneurons. E) Parvalbumin-IR and LacZ staining shows that same populations of parvalbumin-IR cells are V1-derived interneurons. F) Superimposition of calbindin, parvalbumin, and LacZ (V1) immunostaining.
**Figure 11. Labeling in En1-Cre / Thy1-YFP mice.** Low magnification images of a P15 spinal cord section from the En1-Cre / Thy1-YFP animal (line 15). YFP-expressing V1 INs appear in green and the section was also immunostained for calbindin-immunoreactivity (CB-IR, Cy5, white). **A)** Distribution of YFP V1 cells. **B)** Higher magnification of YFP labeled V1 INs showing filling of the cell body, dendrites and axons. **C)** Calbindin-IR cells in the same section as in A. Note the Renshaw cells at the ventral most border. **D)** Superimposition of calbindin-IR and YFP cells.
Figure 12. Labeling in En1-Cre / R26-tdTomato mice. Low magnification images of a spinal cord section from the R26/tdTomato animal. The tdTomato transgenic mouse line shows V1 cells in red. The section was dual immunolabeled for calbindin-immunoreactivity (CB-IR, Cy5, white) and NeuN-immunoreactivity (NeuN-IR, FITC, green). A) The distribution of tdTomato labeled V1 cells is identical to that of the other reporter lines. B) Calbindin-immunoreactivity show the ventral cluster Renshaw cells at the ventral border. C) NeuN-IR is a generalized marker of most spinal cord neuron cell bodies. Note the location of the large motoneuron cell bodies delimiting the extent of the motor pools in this lumbar segment. D) Merge of calbindin-IR and tdTomato labeling of V1-INs. All calbindin-IR Renshaw cells are V1 derived and thus tdTomato positive. E) Merge of NeuN and V1-INs. F) Superimposition of the three fluorochromes. Scale bars; 200μm (all images have same magnification).
Animals

All animal procedures were performed according to NIH guidelines and reviewed by the local Laboratory Animal Use Committee at Wright State University under protocol numbers 736 and 738. All transgenic lines were bred at Wright State. Pups were tail clipped for genotyping and feet tattooed before P5 for identification.

Genotyping

All genotyping was carried out with help from Mrs. Maria Berrocal. DNA from tail clips were extracted using Qiagen’s DNeasy® kit. Genotypes were determined by Polymerase Chain Reaction (PCR) with primers reported in the table below. The PCR was carried out using a MyCycler™ Bio Rad thermocycler with HotMaster™ Taq DNA Polymerase (Eppendorf Brinkmann Instruments, Inc) under the following condition: 5 min 95ºC pre-melt step, followed by 35 cycles of 30 sec 95ºC melt, 30 sec 60ºC anneal, and 7 min 72ºC extension. PCR products were analyzed using 2% agarose gel electrophoresis in 1X TBE buffer with ethidium bromide staining.

Table 1. Primer sequences used for PCR and expected PCR products.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>PCR product</th>
<th>Genotype</th>
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| Cre    | Cre 3: (5’ to 3’ ) TAA TCG CCA TCT TCC AGC AG  
        Cre 4: (5’ to 3’ ) CAA TTT ACT GAC CGT ACA C | 1Kb          | WT: No band  
        Mutant: 1kbp                                    |
| GFP/YFP| EGFP-1: (5’ to 3’ ) GAC GTA AAC GGC CAC AAG TT  
        EGFP-2: (5’ to 3’ ) GAA CTC CAG CAG GAC CAT GT | 600bp        | WT: No band  
        Mutant: 500-600bp                                |
| R26-   | oMR9020: (5’ to 3’ ) AAG GGA GCT GCA GTG GAG TA  
        tdTomato| oMR9021: (5’ to 3’ ) CCG AAA ATC TGT GGG AAG TC  
        oMR9013: (5’ to 3’ ) GGC ATT AAA GCA GCG TAT CC  
        oMR9104: (5’ to 3’ ) CTG TTC CTG TAC GGC ATG G | 200bp-300bp  | WT: 297bp  
        Mutant: 196bp  
        Heterozygous: 297 and 196bp                     |
Timed pregnancies: hormonal treatment

To determine embryonic ages with less than a 12 hour error, pregnancies were facilitated using hormonal injections in females and restricting the mating time-schedules. Hormonal treatment also maximized the number of pregnant females by increasing the number of ova released. Two hormones were injected, Pregnant Mare Serum Gonadotropin (PMSG, Calbiochem, LaJolla, CA, USA), which induces follicular development on day 1, and 48 hours later, Human Chorionic Gonadotropin (HCG, Sigma, CG-10., St Louise, MO, USA) which induces ovulation. Both were intraperitoneally injected (5.0 IU) at 12 pm with a 48 hour delay between them. The females were caged with the males 6 hours after the last hormone injection (beginning of the dark period) to ensure fertilization. The following morning, at 8 am, we checked for vaginal plugs. A positive plug was considered E0.5. Females were weighed daily to confirm successful pregnancy.

Tissue preparation and immunohistochemistry

Mice of different postnatal ages (P0, P5 and P15) were anesthetized with Euthasol (2.0 μg/g i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer (PB). After perfusion, spinal cords were dissected, postfixed in 4% paraformaldehyde overnight and cryoprotected in 0.1M PB (pH 7.4) with 30% sucrose and 0.01% sodium azide. Mouse embryos were extracted from similarly perfused pregnant mothers and fixed in toto overnight and then cryoprotected in 30% sucrose. Embryonic developmental stages were confirmed using the Atlas of Mouse Development (Kaufman, 2005). Histological sections from postnatal spinal cord or
embryos were obtained using a freezing sliding microtome, a vibratome or a cryostat depending on section thickness and age of the preparation. We always used indirect immunofluorescence methods to reveal the proteins of interest. The exact method and antibodies used are detailed in each of the specific aims.

**Analyses**

Immunolabeled sections were analyzed using a Olympus FV 1000 confocal microscope or with epifluorescence in a Neurolucida system. Image analysis of confocal images was done with Fluoview (Olympus), ImagePro (Media Cybernetics) and Neurolucida software (Microbrightfield), a neuron tracing and neuron plotting system. Cell counts and plots were done on “live” epifluorescence in an Olympus BX51 microscope equipped with a motorized stage (Luld electronics, Harborne, NY) and coupled to a digital camera (Microfire, Optronics, Goleta, CA). Statistical analyses were performed using SigmaStat (version 3.1, Jandel).

**Figure composition**

Figures were composed using CorelDraw (ver. 12.0) and graphs in Sigma Plot (ver. 9.0, Jandel). Image modifications for presentation such as, adjusting contrast and brightness were done in Image Pro Plus (ver. 5.0 Media Cybernetics, Bethesda, MD) and preserved all the information content in the original images. Some images were sharpened using a “high gauss” filter. Quantification was always carried out in original unprocessed images.
AIM 1

DETERMINATION OF BIRTHDATES OF DIFFERENT POPULATIONS OF V1-DERIVED INTERNEURONS
INTRODUCTION

The development of spinal cord locomotor circuits depends on the differentiation of the many types of ventral horn interneurons (INs) that modulate motor output (reviewed in Jankowska, 1992; 2008; Brownstone and Bui, 2010). These derive from just a few embryonic subtypes classified according to their early transcription factor expression and origins from specific groups of progenitor cells (Goulding and Lamar, 2000; Briscoe et al., 2000; Goulding and Pfaff, 2005; Goulding, 2009). The mechanisms by which the variety of adult interneuronal circuits emerge from just a few progenitors and subclasses of INs in the embryo are unknown.

Spinal cord INs derive from ten progenitor domains. Six dorsal (pd1 to pd6) and four ventral (p0, p1, p2, and p3) that give rise respectively to dl1 to dl6 dorsal and V0, V1, V2, and V3 ventral embryonic INs, respectively (Goulding, 2009). Motoneurons are generated in an independent ventral domain (pMN), located between p2 and the ventral most p3. Although some premotor INs can be originated from dorsal domains (for example medial laminae V/VI GABAergic neurons, Wilson et al., 2010), many INs that target monosynaptically motoneurons are derived from ventral groups.

V1-derived interneurons (V1-INs) are characterized by the expression of the transcription factor engrailed-1 (En1). Their differentiation potential seems restricted to development of ventrally and laterally located inhibitory INs that extend axons that project ipsilaterally (and that initially take an ascending course). V1 axons frequently make synapses directly onto motoneurons, in addition to other lamina VII neurons (Saueressig et al., 1999; Alvarez et al., 2005). In the adult, all Renshaw cells and some IaINs derive from V1-INs (Sapir et al., 2004; Alvarez et al., 2005; Siembab et al.,
Although Renshaw cells and IaINs share some common properties (they are inhibitory INs with ipsilateral projections), their functionality, connectivity and properties are very different in the adult. In the neonate, V1-derived Renshaw cells and IaINs also display different neurochemical phenotypes and connectivity with motoneurons, primary afferents and in between them (Wang et al., 2008; Siembab et al., 2010). This prompts the question of when they start to differentiate within the V1 group and what factors are involved in determining their fate.

One mechanism of differentiation that is relatively widespread in brain regions with laminar organization (cortex, retina) is based on the time in which cells become postmitotic (reviewed in the background section). Within a single group of neurons their time of generation influences their migration and final location not only in laminar structures but also in brain nuclei (e.g. rostral vs. caudal hypothalamic gonadotropin-releasing hormone neurons: Jasoni et al., 2009). Birth-date can also influence neurochemical phenotype and connections (e.g., different types of amacrine cells in the retina: Voinescu et al., 2009). In the spinal cord it is known that motoneuron birth-date influences their columnar localization and axonal projections (Hollyday and Hamburger, 1977; Sockanathan and Jessell, 1998).

Less is known on the role of birth-dates for the specification of spinal INs. Early birth-dating studies using tritiated thymidine suggested that IN neurogenesis from ventral progenitors occurs earlier than from dorsal progenitors (Nornes and Das, 1974; Nornes and Carry, 1978), but it is not clear if birth-date is of any relevance for the differentiation of different subclasses of INs from single progenitor domains. Recent studies in zebrafish and in mouse analyzed the derivation of IN subtypes from the p2
domain (V2). In the adult zebrafish spinal cord, there are fewer IN subtypes than in mammals, and these are more closely related to their embryonic counterparts (Goulding, 2009). One class of excitatory IN (CiD) corresponds to V2a INs according to transcription factor expression (Alx the homolog of mammalian Chx10). This group is divided into early and late born cells, and each respectively ends up being located more dorsally or more ventrally in the spinal cord and becomes recruited during fast or slow movements (Kimura et al., 2006). Thus, within a single class of adult INs (CiDs) birthdate can impose a gradient of connectivity and function. However, the p2 domain is also known to generate different classes of INs, the V2a excitatory and the V2b inhibitory groups, each characterized by a different transcription factor. This division of the V2 lineage seems to occur in both mice and zebrafish at the time of neurogenesis and involves a notch-delta lateral signaling mechanism that does not necessitate of temporal differences in V2a and V2b generation (Peng et al., 2007; Kimura et al., 2008). In this thesis, which is contemporary to all these recent studies, we will test the hypothesis that a different mechanism is at work in the p1 progenitor domain, that is that Renshaw cells and IaINs derived from this domain have different birth-dates.

A suitable method to monitor the ‘birth-date” of neurons, meaning the time they exit the cell cycle and become postmitotic, is to inject in pregnant females at different stages during pregnancy bromodeoxyuridine (BrdU). BrdU incorporates during S-phase in the DNA of dividing cells and remains at high concentration in the nucleus only if the cell becomes postmitotic immediately after (Miller and Nowakowski, 1988; reviewed in Taupin, 2007). To find the specific “birth-dates” of V1-derived Renshaw cells and IaINs we pulse-labeled embryos in pregnant females with BrdU at embryonic
ages from E9.5 to E12.5. Previous studies suggested that V1-INs exit the progenitor zone and start to differentiate between E9 and E12 (Matise & Joyner, 1997; Saueressig et al., 1999). Analyses were carried out in P15 spinal cords from these animals because at this postnatal age, the phenotypic features that distinguish subgroups of V1-derived INs are already well established (Siembab et al., 2010). We used two different transgenic mouse lines expressing genetic markers for the V1 lineage combined with a number of histochemical criteria to distinguish Renshaw cells from IaINs (Alvarez et al., 2005; Siembab et al., 2010). In the Tau-LacZ line we distinguished timing of BrdU incorporation between calbindin-IR and parvalbumin-IR V1-derived INs, while in the Thy1-YFP line we distinguished between Renshaw cells and IaINs according to their distinct synaptic inputs at P15 (see Siembab et al., 2010).

MATERIALS AND METHODS

Animals

Timed-pregnant females were obtained as explained before. All procedures were carried out according to NIH guidelines and were approved by WSU LACUC.

BrdU injections

Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) is a thymidine analog that incorporates into dividing cells during S-phase (Fig.13). BrdU has to be administered in the right dose to avoid lethal effects or morphological or functional alterations (Taupin et al., 2007; Kolb et al., 1999). NeuN-labeling of P15 spinal cords indicated that the dose chosen for this study (60 mg per Kg weight) did not produce alterations in the size
of the gray matter or cell numbers, suggesting that this dose of BrdU does not affect spinal cord neurogenesis or morphogenesis.

Knowledge of the time course of BrdU incorporation in tissues is important for correct interpretation. Injected BrdU is metabolized through dehalogenation if not integrated into DNA. In the adult, BrdU is available for labeling new born neurons with a half-life of around 2 hours, after which there is an abrupt drop in concentration. If we assume similar availability in fetal tissue and consider that the cell cycle length is of approximately 12-14 hours and the S-phase lasts around 4 hours, a single injection will label only cells that enter S-phase during the 2 hours of BrdU availability after the injection (Taupin, 2007; Packard et al., 1973). Therefore, pulse labeling with a single injection increases timing accuracy, but only labels a small percentage of cells.

The animals were injected with BrdU (Sigma Aldrich, St. Louis, MO, 15mg/ml, 60mg/kg of body weight) diluted in 0.9% NaCl and 0.007% NaOH. BrdU injections were made at five different ages, E9.5, E10.5, E11.5, E12.0, and E12.5. E9.5 is just after neural tube closure in the lumbar region. Previous studies from our laboratory (Maria Berrocal and Francisco J. Alvarez, unpublished) have shown that that after E12.5 there is no BrdU incorporation in V1-INs. All BrdU injections were administered intraperitoneally to pregnant females at 12 pm. Fourteen *En1-Cre/Tau-LacZ* and ten *En1-Cre/Thy1-YFP* pregnant females were injected at different embryonic ages.

*Tissue preparation*

Mice pups from litters treated with BrdU in embryo were anesthetized at P15 with Euthasol (2.0 μg/g i.p.) and perfused transcardially with 4% paraformaldehyde in
0.1M phosphate buffer (PB). After perfusion the spinal cords were dissected, postfixed overnight in 4% paraformaldehyde and cryoprotected in 0.1M PB (pH 7.4) with 30% sucrose and 0.01% sodium azide. The spinal cords were stored in this solution at 4°C.

**Immunolabeling and analysis of V1-INs pulse-labeled with BrdU.**

Fourteen *En1-Cre/Tau-LacZ* and ten *En1-Cre/Thy1-YFP* P15 animals were used to study the distribution of BrdU pulse-labeled V1-INs at five different ages (E9.5, E10.5, E11.5, E12.0 and E12.5). For each line/age one timed-pregnant female was injected and all animals in the litter expressing genetic markers for V1 INs analyzed. One exception was E10.5 in which two females were injected with BrdU. From these litters we analyzed 3 animals per age in the *En1-Cre/Tau-LacZ* line (except for E12.5 in which two animals were analyzed) and 2 animals per age in the *En1-Cre/Thy1-YFP*.

Fifty micron thick sections were obtained in a freezing sliding microtome from the upper (2 and 3) and lower (4 and 5) lumbar segments and processed free-floating. Sections from *En1-Cre/Tau-LacZ* animals were quadruple immunolabeled for calbindin, parvalbumin, β-galactosidase and BrdU. Sections from *En1-Cre/Thy1-YFP* were triple immunolabeled for calbindin, YFP and BrdU. All spinal cord sections were blocked with normal donkey serum diluted 1:10 in 0.01 M PB saline (pH 7.4) with 0.1% or 0.3% Triton X-100 (PBS/Tx) and then incubated overnight with primary antibodies diluted in PBS/Tx. LacZ expression in the *En1-Cre/ Tau-LacZ* was revealed with chicken polyclonal antibodies against β-galactosidase (β-gal, 1:500, AbCam Inc., Cambridge, MA). Yellow Fluorescent Protein (YFP-IR) in the *En1-Cre/Thy1-YFP* was enhanced using an antibody against Green Fluorescent Protein (anti-GFP polyclonal
sheep diluted 1:800, Biogenesis, Brentwood, NH or chicken polyclonal diluted 1:5000, Aves Labs, Tigard, OR). The anti-β-gal antibody was combined with rabbit polyclonal antibodies against calbindin (calbindin D28-K, 1:500, Swant, Bellinzona, Switzerland) and a mouse monoclonal against parvalbumin (1:500, Chemicon, Temecula, CA). The anti-GFP antibody was combined only with rabbit anti-calbindin antibodies (to determine Renshaw cell contacts on YFP labeled V1-INs). The primary antibodies used and their specificity are summarized in Table 2 below.

<table>
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<tr>
<th>Antibody name</th>
<th>Type</th>
<th>Host-species</th>
<th>Dilution</th>
<th>Company</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin D28K</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Swant, Bellinzona</td>
<td>No labeling in KO tissue</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:500</td>
<td>Chemicon</td>
<td>No labeling in KO tissue</td>
</tr>
<tr>
<td>β-gal</td>
<td>Polyclonal</td>
<td>Chicken</td>
<td>1:1000</td>
<td>Abcam Inc.</td>
<td>No labeling in animals with no reporter expression</td>
</tr>
<tr>
<td>GFP</td>
<td>Polyclonal</td>
<td>Chicken</td>
<td>1:5000</td>
<td>Aves Labs</td>
<td>No labeling in animals with no reporter expression</td>
</tr>
<tr>
<td>BrdU</td>
<td>Monoclonal</td>
<td>Rat</td>
<td>1:5000</td>
<td>Sigma Aldrich</td>
<td>No labeling in untreated animals</td>
</tr>
</tbody>
</table>

Immunoreactive sites for β-gal were revealed with a biotinylated donkey anti-chicken antibody (1:100, Jackson InmunoResearch, West Grove, PA) followed by Alexa-405 conjugated streptavidin (Invitrogen, Carlsbad, CA) and YFP-immunoreactive sites were revealed using secondary antibodies conjugated to fluorescein-isothiocyanate (FITC, 1:100; Jackson InmunoResearch). Calbindin was revealed with FITC-conjugated antibodies and parvalbumin using cyanine-5 (Cy5).
conjugated secondary antibodies (all donkey raised and diluted 1:100 in PBS/Tx; Jackson ImmunoResearch) in combination with Alexa 405 β-gal immunofluorescence. Calbindin was revealed with Cy5-conjugated antibodies in combination with FITC YFP-immunofluorescence. The Cy3 red channel was reserved to immunodetect BrdU in all preparations in a follow-up second immunostain. In preliminary experiments we found that treatments to reveal BrdU-immunoreactivity damage the antigenicity for calbindin, parvalbumin, β-gal and YFP and therefore these immunostains were performed before BrdU immunostaining.

For BrdU immunolocalization the DNA was denatured with increasing concentrations (1 to 2N) of hydrochloric acid (HCl) at increasing temperatures (4°C to 37°C). The acid was washed immediately at room temperature with borate buffer (0.1M) and then 0.1M PBS/Tx. Finally the sections were incubated overnight with a rat anti-BrdU monoclonal antibody (dilution 1:5,000; AbCam Inc. Cambridge, UK). BrdU immunoreactivity was revealed with secondary antibodies against rat IgGs coupled to cyanine 3 (Cy3; dilution 1:50 to 1:100 in PBS/Tx, Jackson ImmunoResearch). After all immunoreactions were done the tissue sections were washed in 0.01 M PBS and mounted on gelatin-coated or Histobond (VWR, West Chester, PA, USA) slides and cover-slipped with Vectashield (Vector, Burlingame, CA).

Classification of BrdU cells

BrdU was detected using immunohistochemistry and we distinguished strong and weak nuclear labeling (Fig. 14E, F). In strongly labeled cells, two-thirds or more of the nuclear area was stained homogeneously and these cells were interpreted as cells
that exited the cell cycle immediately after BrdU incorporation (Fig.13). In this case, BrdU was not diluted by further divisions as nuclei of these cells were almost fully covered with strong staining. In contrast, weakly labeled cells appeared to have speckled labeling within the nuclei. Weakly labeled cells can result from: 1) Dilution of BrdU content through further divisions and DNA replication cycles, 2) DNA repair mechanism in some cells, 3) weak incorporation of BrdU during the pulse, for example if a cell enters S-phase at the end of the period of BrdU availability when BrdU concentration diminishes, 4) BrdU incorporation at different phases of S-phase with different chromatin organization (Ferreira et al., 1997). Thus, it is difficult to be certain about the exact significance of weakly labeled cells. We analyzed strong and weakly labeled cells in all our analyses, but we only present data for strongly BrdU labeled cells.
Figure 13. BrdU incorporation into DNA during S-phase of the cell cycle. BrdU is only incorporated during DNA replication. If the cell leaves the cell cycle right after BrdU incorporation the nuclei appears strongly labeled, while if the cells goes through further divisions or there was not enough BrdU available in the system it appeared as weakly labeled with BrdU.
BrdU strong

BrdU weak

Mitosis (Cell division)

Start of the cell cycle

Cell enlarges and makes new proteins

DNA replication

BrdU incorporation

Restriction point (cell decides whether to commit itself to complete the cycle)

12-14 Hours

Cell rests (G0)
Analysis

The numbers and positions of V1-INs with or without calbindin or parvalbumin that incorporated BrdU at each embryonic age were plotted on a Neurolucida system (MicroBrightField, Colchester, VT) coupled to an epifluorescence BX50 Olympus microscope with a motorized stage (Luld electronics, Harborne, NY) and z-axis encoder and imaged “live” with a digital color camera (Microfire CCD, Optronics, Goleta, CA). V1-INs with and without BrdU labeling were counted and their positions plotted on outlines of the spinal cord sections obtained first at low magnification. To identify YFP-V1 INs receiving calbindin contacts (i.e., IaINs) we obtained higher resolution confocal images (at 10X, 20X and 60X) using an Olympus FV100 system. Image confocal stacks were obtained through the whole tissue section and fed into Neurolucida for counting and plotting. We analyzed 10 ventral horns per animal in lower and upper lumbar levels. Three animals were analyzed per age in the En1-Cre/Tau-LacZ line (with the exception of E12.5 in which only two animals were studied) and two animals per age in the En1-Cre/Thy1-YFP. From the Neurolucida cellular plots we estimated: 1) the percentage of V1 INs labeled with nuclear BrdU in the En1-Cre/Tau-LacZ and En1-Cre/Thy1-YFP lines, 2) the percentage of calbindin- or parvalbumin-immunoreactive V1 INs that incorporated BrdU at each embryonic age using the En1-Cre/Tau-LacZ line and 3) the percentage of V1-derived Renshaw cells and IaINs with BrdU in the En1-Cre/Thy1-YFP line.

The data is presented for individual animals as well as average percentages obtained by pooling together the animal averages from each line or the two animal lines.
when possible (n = 4 to 5 animals). Pooled averages were compared using one-way ANOVA (SigmaStat ver. 2.0, Jandel). Significance was set at p<0.05.

**Dorso-ventral distribution of BrdU labeled V1-derived interneurons.**

The thoracic segments of 12 spinal cords from P15 En1-Cre/ Tau-LacZ animals were used to analyze possible differences in dorso-ventral location of BrdU labeled V1-INs. The animals were pulsed labeled with BrdU at E9.5, E10.5, E11.5 and E12.5 (n=3 animals per age). Thoracic segments were used in this analysis because in this region the ventral horns preserve better the original embryonic orientation of the grey matter. Spinal cord sections were double immunostained as previously described. The cells were counted and plotted in the Neurolucida system. The positions of BrdU labeled V1-INs were analyzed in a grid of five dorso-ventral 100 μm bins dividing the ventral horn in different dorso-ventral regions. We analyzed 10 ventral horns per animal.

**Internal controls for the timing of BrdU injections and BrdU incorporation.**

The spinal cord at thoracic levels contains five groups of cholinergic neurons that can be immunolabeled with antibodies against choline acetyltransferase (ChAT) and have known birth-dates (Barber et al., 1984; Phelps et al., 1988; Phelps et al., 1991; Barber et al., 1991). To confirm that BrdU injections were delivered at the correct estimated times in pregnant females, we first analyzed the dorso-ventral distribution of all BrdU labeling and then the labeling in different groups of ChAT-immunoreactive neurons. Thoracic spinal cord sections from all the animals used in the study were double immunolabeled with a goat polyclonal ChAT antibody (diluted 1:500, Milipore,
Billerica, USA) and the rat monoclonal BrdU antibody (1:5000, AbCam Inc. Cambridge, MS, USA) using the same method described before. Immunolabeling for ChAT using FITC conjugated secondary antibodies was followed by DNA denaturation and BrD-U immunohistochemistry revealed with Cy3-conjugated antibodies. The percentage of strongly BrdU-immunolabeled in each cholinergic group was estimated using the Neurolucida plotting system. We analyzed 10 to 20 ventral horns in all the En1-Cre/Tau-LacZ and En1-Cre/Thy1-YFP animals prepared for study. Litters in which BrdU was preferentially incorporated into the inappropriate cholinergic group according to time of embryonic injection were discarded. We only discarded 2 animals for one litter of the En1-Cre/ Tau-LacZ line and 3 animals from one litter in the En1-Cre/Thy1-YFP line. This represents an 80-85% success rate with our method of timing pregnancies. However, given that males and females are caged together for 12 hours before checking plugs we should expect ±0.5 day error in our estimated times.

Figure composition

All images for presentation were obtained with an Olympus FV1000 confocal microscope. Triple or quadruple color immunofluorescent preparations were first imaged at low magnification (10x1 or 20x1). Representative cells were selected for obtaining series of confocal optical sections throughout their cells bodies and dendrites at high magnifications using a 60x1 oil objective (N.A. 1.35) and a z-step of 0.5μm. Figures were composed using CorelDraw (ver. 12.0) and graphs in Sigma Plot (ver. 9.0, Jandel).
RESULTS

BrdU pulse-labeled neurons in the spinal cord from E9.5 to E12.5

In P15 mouse spinal cords, we found overall a ventral to dorsal and lateral to medial pattern of IN labeling with BrdU delivered at increasingly older ages from E9.5 to E12.5 (Fig. 14). This pattern is similar to that described before using $^3$H-thymidine injections in the mouse (Nornes and Das, 1974; Nornes and Cary, 1978). In the lumbar cord at E9.5, mostly motoneurons in LIX together with a few ventral INs clustered around motor pools incorporated strong BrdU labeling (Fig. 14A). At E10.5 (Fig. 14B), there were fewer motoneurons strongly labeled with BrdU and the number of INs increased. BrdU-labeled INs are mostly located ventrally in LVII and LVIII. From E11.5 to E12.5 (Fig. 14C and D) strongly labeled INs were found in more medial and dorsal locations. At E11.5 and later, we found no BrdU in motoneurons and at E12.5, strongly labeled INs were restricted to the dorsal horn. These distributions of BrdU-labeled cells in the spinal cord agrees well with the known gradient in cell generation indicating a correct estimate of timed pregnancies and embryonic injections.

Analysis of BrdU incorporation in cholinergic groups

To ensure that the estimated embryonic times for BrdU injections were correct, we further analyzed BrdU labeling of cholinergic neurons in thoracic segments in all litters that generated. There are five types of ChAT-immunoreactive neurons at thoracic levels. These include ventrally located somatic motoneurons (MN), intermedio lateral horn motoneurons (IML), partition cells (PC), central canal cluster cells (CC) and dorsal horn INs (DH) (Barber et al., 1984). Each group exhibits a characteristic birth-
Figure 14. Distribution of Bromo-deoxyuridine (BrdU) labeled nuclei in spinal cord hemisections at four different ages. Low magnification (A, B, C, D) and high magnification (E, F) confocal images of lumbar spinal cord hemi-sections immunolabeled with BrdU (Cy3, red) and either β-galactosidase (β-gal; 405, E) or Yellow Fluorescent Protein (YFP; FITC, F) in En1-Cre/Tau-lacZ (E) and En1-Cre/Thyl-YFP (F) P15 animals. Pregnant females were injected with BrdU at gestation days 9.5, 10.5, 11.5, and 12.5. The dotted white lines delineate the border between the white and grey matter and continuous white lines outline the spinal cord. The border between lateral lamina IX and the rest of the spinal cord is also indicated. A) Distribution of BrdU-immunoreactive (BrdU-IR) nuclei in animals injected at E9.5. Both motoneurons (solid white arrows) and some ventral interneurons display strongly labeled nuclei. B) Distribution of BrdU-IR in animals injected at E10.5. The BrdU pattern is restricted to a few very ventral interneurons and some more lateral and dorsal interneurons. C) BrdU-IR in animals injected at E11.5 showing almost no ventral labeling. Most labeled nuclei are located close to the central canal and dorsally. D) Distribution of BrdU-IR nuclei in an animal injected at E12.5 showing only dorsally labeled nuclei. E) High magnification confocal image of BrdU-IR nuclei in cells from an En1-Cre/Tau-lacZ animal. Some nuclei of V1 cells (β-gal positive in blue) show strong (solid white arrows) or weak (open arrow) immunolabeling. F) BrdU-IR nuclei in En1-Cre/Thyl-YFP animal injected at E10.5 (solid white arrows, strong labeling; open white arrows, weak BrdU-IR). Scale bars; 200μm in A (B, C, and D have same magnification); 10μm in E (F has same magnification).
date peak in rats tested with $^3$H-thymidine (Phelps et al., 1988); MN and IML are generated simultaneously around E11, while PC, CC, and DH peak respectively at E12, E13 and E14. Thus, we expected that the proportions of strongly labeled BrdU cells in each cholinergic group should confirm the sequence of embryonic BrdU injections. Direct comparison of exact ages with the rat is not possible since spinal cord neurogenesis occurs slightly earlier in the mouse. In the mouse, 90% of MNs are generated between E9 and E10.5 (Sims and Vaughn, 1979; Holley et al., 1982; Wentworth, 1984). This suggests that generation of other cholinergic neurons in the mouse might also occur 2 days earlier. Consistent with this most animals pulse-labeled with BrdU at E9.5 displayed labeling mostly in somatic (MNs) and visceral motoneurons (IMLs), while at E10.5 MN labeling had decreased and BrdU incorporation was predominant in PC cells (Figs. 15 and 17). At E11.5, the largest BrdU labeled group were CC cells and at E12 many CC cells were still labeled while the proportion of DH cells increases (Figs 15, 16 and 17). Finally, at E12.5 only DH INs incorporated BrdU (Figs. 16 and 17). Thus, the generation sequence of thoracic cholinergic INs in the mouse spinal cord is in good agreement with the pattern described in the rat with a 24-36 hour difference in different cholinergic subgroups. Moreover, the labeling patterns confirmed estimated embryonic times for BrdU pulse-labeling in the majority of animals. Two out of twelve BrdU injected litters in the En1-Cre/Thy1-YFP and one out of sixteen in the En1-Cre/Tau-LacZ were discarded because the pattern of incorporation of BrdU in cholinergic cells did not match the timing of the BrdU injections. This error was most likely due to pregnancy dating errors and these animals were not analyzed further.
Figure 15. Distribution of BrdU-labeling in cholinergic neurons in thoracic segments after BrdU injections (E9.5, E10.5 and E11.5). A, C, and E) Low magnification and B, D, and F) high magnification confocal images of P15 mouse thoracic spinal cord sections dual immunolabeled from BrdU (Cy3, red) and ChAT (Choline Acetyltransferase, green, FITC). BrdU injections were done at E9.5 (A and B), E10.5 (C and D) and E11.5 (E and F). Yellow boxes in A, C and E indicate the areas shown at higher magnification in B, D and F. Dotted lines delineate the border between the white and grey matter. The central canal is also indicated in the center. In the high magnification images (B, D and F) solid white arrows indicate cells strongly labeled with BrdU, while open arrows point to weakly labeled cells. A, B) At E9.5 strong BrdU-labeling appears mostly on motoneurons and intermedio-lateral motoneurons (IML). C, D) At E10.5 fewer motoneurons are strongly labeled with BrdU and mostly partition cells are strongly labeled (PCs, large cholinergic interneurons close to the central canal). E, F) At E11.5, we can still detect some PC cells strongly labeled; however, the smaller central canal cells (CC) are the predominant group labeled at this age. Scale bars; 200μm in A (C and E have the same magnification); 100μm in B (D and F have the same magnification).
Figure 16. Continuation on the distribution of BrdU-labeling in cholinergic neurons in thoracic segments after BrdU injections (E12.0 and E12.5). G and I) Low magnification and H and J) high magnification confocal images of P15 mouse thoracic spinal cord sections dual immunolabeled from BrdU (Cy3, red) and ChAT (Choline Acetyltransferase, green, FITC). BrdU injections at E12.0 (G and H) and E12.5 (I and J). Labeling is as in Figure 15. At these ages most BrdU-IR cells are located in the dorsal horn. However, at E12.0 (G and H) we can still detect some CC cells generated near the central canal, while none of the CC cells at E12.5 are labeled. Scale bars: 200μm in G (I has the same magnification); 100μm in H (J has the same magnification).
Figure 17. Quantification of BrdU incorporation in thoracic cholinergic neurons. 
A) Diagram of the thoracic spinal cord showing the location of the five different types of cholinergic neurons described previously in the rat (dotted rectangles). B) Low magnification confocal image of a P15 mouse thoracic spinal cord section immunolabeled for ChAT (green, FITC). Yellow dotted line delineates the border between the white and grey matter. White dotted boxes indicate the locations of the cholinergic cells. C and D) Percentage of ChAT positive interneurons of each type strongly labeled with BrdU at the different ages in the litters obtained from En1-Cre/Tau-lacZ (C) or form En1-Cre/Thy1-YFP (D) mouse lines. Each bar shows the average value of all the animals analyzed at each age. Data sample in C: N=5 animals at E9.5, N=7 animals at E10.5 and N=4 animals at E11.5 and E12.5. In each animal 10 ventral horns were counted at thoracic level. Error bars indicate S.E.M. Data sample in D, two animals were analyzed at each embryonic age. Bars indicate the average between these two animals. No error bars are shown. In both cases, we can observe that at E9.5 motoneurons (MN and IML) are the most predominant labeled group. The most abundant group born at E10.5 are the partition cells; while at E11.5 are the central canal cells followed by the dorsal horn cells at E12.0 and E12.5. However, while at E12.0 we can still detect CC, at E12.5 there is no other cell type generated besides the dorsal horn cells. Note: error bars increase when the number of cells sampled per section is very low (1 or 2 cells), that is the case for the relatively rare dorsal horn cholinergic neurons. E) Cell type distribution according to age. Grey boxes represent the cell type most abundant at each age studied. White boxes indicate fewer strongly BrdU labeled cells. A dash indicates no cells were found strongly labeled with BrdU. This pattern of BrdU incorporation in our litters confirms the expected birth-dates of cholinergic neurons and demonstrates that injections times correspond with the expected values.
A. Cholinergic neurons localization at thoracic levels in the mouse spinal cord

- Dorsal Horn (DH)
- Intermedio lateral (IML)
- Partition cells (PC)
- Central canal (CC)
- Motoneurons (MN)

B. Mouse spinal cord at thoracic levels

C. En1CRE/Tau: Cholinergic neurons with BrdU at thoracic levels

D. En1CRE/Thy1: Cholinergic neurons with BrdU at thoracic levels

E. Table showing each cell type distribution according to the age

<table>
<thead>
<tr>
<th></th>
<th>E9.5</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E12.0</th>
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<td>MN</td>
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</table>
Distribution of BrdU labeling in the V1 population

V1-derived INs were identified in each transgenic line by either nuclear β-gal or YFP labeling (Fig. 14E and F). BrdU labeling was confined to the cell nucleus. Sometimes most of the cell nucleus was covered with BrdU immunoreactivity, another times only a few speckles were found. Only data on strongly labeled cells (>75% nuclear coverage) is presented below. V1-derived INs were located ventrally (Figs. 10, 11, and 12). As previously reported (Siembab et al., 2010) there were approximately 25% less V1 cells in En1-Cre/Thy1-YFP animals compared to En1-Cre/Tau-LacZ (Fig. 18A). We compared upper lumbar (L2 and L3) segments to lower lumbar (L4 and L5) regions because the possibility of rostro-caudal differences in cell number and/or generation time. No differences between upper and lower lumbar regions were noted in total number of V1 INs in the Tau/LacZ and Thy1/YFP lines (Fig. 18A). Similarly there was little variation in the number of V1-derived INs expressing calbindin or parvalbumin (Fig. 18B), or classified as Renshaw cells or IaINs (Fig. 18B and 18C; this is more likely because analysis in upper lumbar segments were biased towards lumbar 3 sections). The only exception was a dorsally located calbindin-IR V1 IN characterized by a very large size soma and dendrites (“Big calbindin”). This cell type was only found in lumbar 2 and 3 levels (Fig. 18B).

Analyses of BrdU incorporation confirmed that V1-INs incorporate BrdU from E9.5 to E12, with almost no V1 cells pulse-labeled by BrdU at E12.5 (Figs. 19 and 20). Quantitative analyses suggested that there are two peaks of V1-IN generation, one at E10.5 and the other at E12.0 with lower BrdU labeling at E9.5 and E11.5. This result was consistent in different animals, lines and in different lumbar segments (Figs. 21 and
Figure 18. Comparison of V1-INs number examined in En1-Cre/Tau-lacZ and En1-Cre/Thy1-YFP animals and at different lumbar levels. A) Average total number of V1-derived interneurons sampled per ventral horn in different animals from each mouse line and lumbar region. Fourteen animals were analyzed in the En1-Cre/Tau-lacZ lines. Ten animals were analyzed in the Thy1-YFP line. Ten ventral horns were counted per animal in each lumbar segment, lower (L4/L5) and upper (L2/L3). No significant differences were detected in cell numbers per ventral horn between lower and upper lumbar segments within each line (t-test, P>0.05). However, between lines there is a significant 25% decrease in the number of V1-INs detected per ventral horn in the En1-Cre/Thy1-YPF line (t-test, P<0.05). B) Average numbers of different V1 cell types detected in En1-Cre/Tau-LacZ animals: V1-derived calbindin-IR (V1-CB), Renshaw cells (RC), V1 big-calbindin-IR (V1-big CB), and V1-derived parvalbumin cells that are not Renshaw cells (non-Renshaw cells, V1-PV). Differences in between lumbar segments are not significant except in the case of big CB-IR cells that are not found in lumbar 4 and 5 segments. C) In the Thy1-YFP line only two cell types were distinguished: Renshaw cells (RC) versus Ia inhibitory interneurons (IaINs). Similar numbers were detected in different lumbar segments (t-test, P>0.05). All error bars indicate standard error of the mean (S.E.M).
A. Total number of V1/INs in Tau/LacZ and Thy1/YFP

B. Total number of cells in Tau/LacZ

C. Total number of cells in Thy1/YFP
Figure 19. Distribution of BrdU-immunoreactivity in V1-derived interneurons in *En1-Cre/Tau-lacZ* P15 mice pulse-labeled with BrdU at 4 different ages. A, D, G, and J) Distribution of V1-derived interneurons in the P15 ventral horn immunolabeled against β-gal (Alexa 405, blue). B, E, H, and K) Pattern of BrdU labeling in the same sections (Cy3, red). Solid white arrows point to V1-derived interneurons strongly labeled with BrdU while open arrows point to non-V1 interneurons cells strongly labeled with BrdU. C, F, I, and L) Superimposition of BrdU and nuclear LacZ labeling. Females were injected at 4 embryonic ages. A-C: E9.5; D-F: E10.5; G-I: E11.5; J-L: E12.5. The dotted lines indicate the border between the white and grey matter in the ventral spinal cord. The images clearly show how the BrdU labeling starts laterally and ventrally and moves medially and dorsally as the development progresses. By E12.5 there are very few ventral V1 neurons strongly labeled with BrdU. Scale bar; 100 μm in A (all images have the same magnification).
Figure 20. Distribution of BrdU-immunoreactivity in V1-derived interneurons in En1-Cre/Thy1-YFP P15 mice pulse-labeled with BrdU at 4 different ages. A, D, G, and J) Distribution of YFP-labeled V1-derived interneurons (green, FITC + YFP) in the P15 ventral horn. B, E, H, and K) Pattern of BrdU labeling in the same sections (Cy3, red). Solid white arrows point to V1-derived interneurons strongly labeled with BrdU while open arrows point to non-V1 interneurons cells strongly labeled with BrdU. C, F, I, and L) Superimposition of BrdU and YFP labeling. Females were injected at 4 embryonic ages. A-C: E9.5; D-F: E10.5; G-I: E11.5; J-L: E12.5. The dotted lines indicate the border between the white and grey matter in the ventral spinal cord. The images show how the BrdU labeling starts laterally and ventrally and moves medially and dorsally in animals injected with BrdU at older ages. By E12.5 there are very few ventral V1 neurons strongly labeled with BrdU. Scale bar; 100 μm in A (all images have the same magnification).
We did not observe differences between the two mouse lines, other than the number of BrdU strongly labeled V1 cells was higher in En1-Cre/Tau-LacZ animals due to the higher number of labeled V1-INs compared to the En1-Cre/Thy1-YFP. When pooling data from both lines together (Fig. 22 E, F), it appears that there are two waves of S-phase BrdU incorporation in V1 INs. One starting at E9.5 and peaking at E10.5 and the second starting at E11.5 and peaking at E12.

Thoracic distribution of V1-derived interneurons labeled with BrdU at different ages.

Kimura et al. (2006) demonstrated in the zebrafish spinal cord that V2a INs generated at different times become located at different dorso-ventral positions in the spinal cord. To test the possibility of a dorso-ventral pattern in V1-derived INs in the mammalian spinal cord we analyzed the position of V1-INs that incorporated BrdU at different embryonic ages using Neurolucida cell plots. Analyses were performed in thoracic segments of En1-Cre/Tau-LacZ animals (Fig. 23). In these animals we labeled all possible V1 INs and we chose thoracic levels because the embryonic dorso-ventral organization seems best preserved at this level in the mature spinal cord. We divided the ventral horn in 5 dorso-ventral bins of 100 μm thickness starting at the central canal level (Fig. 24A), and calculated the percentage of V1-INs strongly labeled with BrdU in each bin. We could not detect a clear pattern consistent with a dorso-ventral sequence of V1-INs generated at different times in the middle bins (Fig. 24B). V1-INs located in the ventral most bin (400-500 μm) were, however, generated only at E9.5 and at E12.5 most V1-INs with BrdU were located in the more dorsal bin.
Figure 21. Quantitative analysis of strong BrdU-immunoreactivity in V1-derived interneurons (data from individual animals). Histograms show the percentage of V1-INs strongly labeled with BrdU in individual En1-Cre/Tau-lacZ (A and B) and En1-Cre/Thy1-YFP (C and D) animal lines divided in lower (L4/L5; A and C) and upper (L2/L3; B and D) lumbar segments. BrdU injections were made at the 5 embryonic ages indicated in the x-axis. The first three digits correspond with litter number (as per our full colony at WSU). After the dot the number represents the animal number in the litter. These numbers were for identification purposes and it allows determination of litters of origin. Variations in the number of labeled cells in each animal were always consistent between upper and lower lumbar segments, suggesting that these differences are intrinsic inter-animal differences perhaps due to slightly more or less advance stages of development of individual animals within litters. Note that in a single litter some animals will contain low and others relatively high BrdU labeling. Despite this variation among animals we can observe among different litters, lines and lumbar segments a consistent increase in the number of BrdU labeled V1 interneurons at E10.5 and E12.0. At E12.5 very few V1-INs were strongly labeled.
A. V1 INs (LacZ+) with BrdU at lower lumbar segments (L4/L5)

B. V1 INs (LacZ+) with BrdU at upper lumbar segments (L2/L3)

C. V1 INs (YFP+) with BrdU at lower lumbar segments (L4/L5)

D. V1 INs (YFP+) with BrdU at upper lumbar segments (L2/L3)
Figure 2. Percentages of strong BrdU incorporation in V1-derived interneurons at different embryonic ages (pooled data). **A and B**) Percentage of V1-INs strongly labeled with BrdU in the En1-Cre/Tau-lacZ mouse. Data sample: N=3 animals at E9.5, E10.5 and E11.5; N=2 animals at E12.5. Error bars in this and following histograms indicate S.E.M. **C and D**) Similar analysis in the En1-Cre/Thy1-YFP mouse line. Each bar represents the average of 2 animals. **E and F**) Percentage of V1-INs strongly labeled with BrdU in both mouse lines pooled together. Each bar represents the average of 5 animals, except for E12.5 where the average comes from 4 animals. In all cases ANOVA analyses showed significant differences among the ages (p<0.001). Post-hoc analyses consistently showed significant differences (p<0.05; asterisks) at all ages with E12.5 and differences between E11.5 and E12. Fewer BrdU V1-interneurons were always detected at E9.5 compared to E10.5, but significant differences were only detected by the post-hoc analysis in the lower lumbar segments. We conclude that these histograms strongly suggest two waves of BrdU incorporation. The first one starts at E9.5 and peaks at E10.5 and the second one starts at E11.5 and peaks at E12.0. The consistency and repeatability of the result among animals (Fig. 21), lines and lumbar segments gives confidence in the strength of this conclusion.
Figure 23. Distribution of strong BrdU labeling in thoracic V1-derived interneurons. P15 thoracic spinal cord sections of En1-Cre/Tau-LacZ animals injected with BrdU at E9.5, E10.5, E11.5 and E12.5 were immunostained with LacZ (green, FITC) and BrdU (red, Cy3). Arrows indicate V1-INs strongly labeled with BrdU. A) Spinal cord section of an animal injected at E9.5 showing BrdU labeling of V1-INs throughout the entire ventral horn. B) At E10.5 we detected the same dorso-ventral pattern of labeling. C) By E11.5 BrdU labeled cells start moving more dorsally however, we can still detect few V1-INs strongly labeled for BrdU in the ventral horn. D) At E12.5 we detected few V1-INs strongly labeled for BrdU and this were generally located relatively dorsal within the V1 population.
Figure 24. Analysis of dorso-ventral distributions of BrdU-labeled V1-derived interneurons in the thoracic spinal cord. Analyses were done in 10 ventral horns of P15 En1-Cre/Tau-LacZ animals injected at 4 embryonic ages with BrdU (E9.5, E10.5, E11.5, and E12.5). A) The ventral horn of the spinal cord was divided in five 100 µm bins and the number of V1-interneurons with or without strong BrdU labeling counted in each bin. The bins were generated from the central canal, starting at the same level of the central canal the first one was denominated 0-100 and from there 100-200 bin, 200-300 bin, 300-400 bin, and 400-500 the most ventral bin. B) Percentages of strongly labeled BrdU V1 cells found in each bin over the total number of BrdU-labeled V1 interneurons. At early embryonic ages, V1-INs strongly labeled with BrdU are distributed in all bins. However, after E9.5 there are no V1-INs labeled with BrdU in the most ventral bin and the percentage of cells labeled in more dorsal bins increased. By E12.5 there are no V1s labeled in the two most ventral bins and the number of cells of the most dorsal bin has considerably increased. Thus, although we do not observe a clear pattern in the middle bins, we can detect a tendency of cells located more ventrally being labeled earlier and cell located more dorsally being labeled with BrdU at later embryonic ages.
V1-derived interneurons expressing different calcium binding.

Two calcium binding proteins, calbindin and parvalbumin, distinguish subclasses of V1-derived INs, namely Renshaw cells and IaINs (Alvarez et al., 2005). In order to analyze BrdU incorporation in calbindin and parvalbumin V1-INs we performed triple and quadruple immunolabeling of spinal cord sections (Fig. 25). For these analyses we used the *En1-Cre/Tau-LacZ* mouse line. Calbindin-immunoreactivity distinguished two groups of V1-INs based on location and morphology. Renshaw cells are small cells located ventrally in the region where motor axons exit from the spinal cord and are found in all lumbar segments analyzed. A proportion of Renshaw cells also express parvalbumin (Fig. 26). A novel group of calbindin-IR V1 cells was found in upper lumbar segments (these segments have not been analyzed in previous studies of V1-derived INs, Alvarez et al., 2005). These cells have large cell bodies extensive dendrites and are located relatively dorsal in LVII within the distribution area of the V1 population (Fig. 27). This novel V1 cell type and their BrdU incorporation was confirmed in *En1-Cre/Thy1-YFP* animals (Figs. 28, 29, 30). Parvalbumin-IR V1-INs were located at all dorso-ventral locations and we excluded from this group Renshaw cells (some also express parvalbumin). Parvalbumin-IR V1-INs were generally large and displayed extensive dendritic trees (Fig. 31).

Quantitative analyses showed that calbindin-IR V1-derived INs were mostly generated between E9.5 and E10.5 (Fig. 32), with the largest percentage generated at E9.5 in both upper and lower lumbar segments. Renshaw cells and big V1 calbindin-IR neurons were labeled with BrdU at E9.5 and E10.5. No BrdU incorporation was
detected in these cells at E11.5, E12.0 or E12.5. A few V1-INs of small size with very weak calbindin and that did not correspond to any of the former groups incorporated BrdU at E11.5 and E12. These cells appear to correspond with groups of V1 INs that are in the process of downregulating calbindin expression in the postnatal spinal cord (Siembab et al., 2010). Parvalbumin-IR V1-derived INs different from Renshaw cells showed a broader generation time window from E9.5 to E12.5 in both lumbar regions (Fig. 32). The percentage and number of parvalbumin-IR V1-INs generated at E9.5 is, however, much lower than the percentage of calbindin-IR V1-INs at that same embryonic age.

In conclusion, V1-derived INs expressing different types of calcium buffering proteins are generated at different embryonic times.

*Renshaw cells are generated first, followed by Ia inhibitory interneurons during much broader window generation times*

The above results suggest that V1-derived Renshaw cells might be generated earlier than V1-derived IaINs. To confirm this hypothesis we directly analyzed BrdU labeling in V1-derived IaINs and compared them to Renshaw cells in the same sections using the *En1-Cre/Thy1-YFP* model. In this model the cell bodies, dendrites and axons of V1-derived cells are labeled with YFP and this allowed us to define V1-derived IaINs as those V1’s receiving inputs from Renshaw cells (Alvarez et al., 2005; Siembab et al., 2010). Renshaw cell axonal inputs were visualized as baskets of axons labeled with YFP and calbindin and profusely contacting the cell soma and proximal dendrites of putative V1-derived IaINs (Fig. 33). In these animals we confirmed that most
Figure 25. BrdU labeling of V1-derived interneurons expressing different calcium-binding proteins in En1-Cre/Tau-lacZ mice spinal cords at P15. The dotted lines delineate the borders between the white and grey matter. CC, indicates the central canal position. A, B, C, D) Low magnification confocal images of lumbar spinal cord hemisections immunolabeled for β-gal (Alexa-405, blue, A), BrdU (Cy3, red, B), calbindin (CB; FITC, green, C) and parvalbumin (PV; Cy5, white, D). This particular animal was exposed to BrdU at E10.5. Ventrally located calbindin-IR V1-derived interneurons correspond with Renshaw cells (RC area). In addition, a few V1-derived calbindin-IR cells were located more dorsally and do not correspond with Renshaw cells. These were more frequent in upper lumbar regions and were divided into large and small cells according to soma size. Large V1 calbindin-IR cells (Big CB) were only detected in upper lumbar regions. Parvalbumin immunostaining is in a population of V1-INs but it also labels many other non-V1-INs. E, F, G, H) Higher magnification confocal images of ventral spinal cord sections showing V1 interneurons with BrdU labeling (E), calbindin-immunoreactivity (F), parvalbumin-immunoreactivity (G) and superimposition of all four fluorochromes (H). Scale bars; 200 μm in A and E (B, C and D same magnification as A; F, G and H same magnification as E).
**Figure 26. BrdU incorporation in Renshaw cells.** High magnification images of Renshaw cells from a P15 Enl-Cre/Tau-lacZ animal injected with BrdU at E10.5. The section was quadruple immunolabeled for LacZ (blue, Alexa-405), calbindin (CB; green, FITC), parvalbumin (PV; white, Cy5), and BrdU (red, Cy3). **A to I** Show images of these four fluorochromes in different combinations. Solid arrow (in C) indicates strong BrdU labeling, while the open arrow points to a weakly labeled nucleus. Calbindin-IR cells in general and Renshaw cells in particular incorporated BrdU at E9.5 and E10.5. These particular images show two Renshaw cells. One of them strongly labeled by BrdU injected at E10.5. The other cell is weakly labeled. These two Renshaw cells expressed various levels of parvalbumin-immunoreactivity (G-I). Scale bars; 10 μm in A (all images have the same magnification).
Figure 27. BrdU incorporation in dorsal and large calbindin-immunoreactive V1-INs in an En1-Cre/Tau-LacZ animal injected with BrdU at E10.5. Medium magnification confocal images of two big calbindin-IR cells immunostained for LacZ (blue, Alexa-405), calbindin (green, FITC) and BrdU (red, Cy3). A-F) Show images of the same microscopic field with each of these three fluorochromes in different combinations. Big calbindin-IR V1 cells are strongly labeled with BrdU at early embryonic ages, between E9.5 and E10.5. This section contains two big calbindin-IR V1 cells, however only one of them is strongly labeled with BrdU. Scale bar; 20μm in A (all images have the same magnification).
Figure 28. BrdU labeling of calbindin-IR V1-derived interneurons in an En1-Cre/Thy1-YFP animal injected with BrdU at E9.5. A, B) Low magnification confocal images of the spinal cord immunolabeled with calbindin (CB; Alexa-405, white; A) and Yellow Fluorescent Protein (YFP; FITC, green; B). The dotted line indicates the boundary between the grey and white matter. C, D, E, F) Higher magnification confocal images of the ventral horn; Calbindin (C), YFP (D), BrdU (Cy3, red; E) injected at E9.5 and superimposition of CB and BrdU (F). Some Renshaw cells and the more dorsal calbindin-IR cell show strong BrdU labeling in their nuclei. G, H, I, J, K, L) High magnification confocal image of the dorsal calbindin-IR V1 interneuron immunolabeled for YFP (G), calbindin (H) and BrdU (I). BrdU labeling is shown superimposed to YFP (J) and calbindin (K), and calbindin and YFP labeling are superimposed in L. Scale bars; 200μm in A (B has the same magnification); 100μm in C (D, E, and F are at the same magnification); 30μm in G (H, I, J, K, L are at the same magnification).
Figure 29. BrdU labeling of calbindin-IR V1-derived interneurons in an En1-Cre/Thy1-YFP animal injected with BrdU at E10.5. A, B) Low magnification confocal image of a P15 spinal cord immunolabeled for calbindin (Alexa-405, white; A) and BrdU (Cy3, red; B) injected at E10.5. The dotted line indicates the boundary between the grey and white matter. Yellow boxes indicate the areas displayed below at higher magnifications (C, D, E and F). The white box (A) indicates two big-calbindin-IR V1 interneurons that are more dorsally located. C, D, E, F) Higher magnification confocal images of the ventral horn labeled with YFP (FITC, green; C), calbindin (D), BrdU (E) and superimposition of YFP and BrdU (F). The dotted line indicates the boundary between the grey and white matter. G, H, I, J, K, L) High magnification confocal image of two big calbindin-IR V1 interneurons (arrows) expressing YFP (G), calbindin-immunoreactivity (H) and containing BrdU (I). Superimposed images of YFP and calbindin-IR (J), YFP and BrdU-IR (K), and calbindin and BrdU (L). Scale bars; 200μm in A (B is at the same magnification); 100 μm in C (D, E, and F are at the same magnification); 30 μm in G (H, I, J, K, and L are at the same magnification).
Figure 30. BrdU labeling of calbindin-IR V1-derived interneurons in an En1-Cre/Thy1-YFP animal injected with BrdU at E12.5. A, B, C) Low magnification confocal images of spinal cord hemi-section immunolabeled with calbindin (CB; 405, white; A), Yellow Fluorescent Protein (YFP; FITC, green; B), and BrdU (Cy3, red; C). The dotted line indicates the boundary between the grey and white matter. D, E, F, G, H, I) Higher magnification confocal images of the ventral horn labeled with calbindin (D), YFP (E), and BrdU (F) injected at E12.5 and superimposition of calbindin and BrdU (G). No calbindin-IR V1 cells are labeled with BrdU at this age. H) V1 interneurons superimposed to BrdU labeling at E12.5 shows only a few V1 interneurons with strong BrdU labeling on their nuclei. I) Superimposition of calbindin and YFP shows that none of these were calbindin-immunoreactive. J, K, L, M, N, O) High magnification confocal images of a dorsal CB-IR V1 interneuron immunolabeled for YFP (G), calbindin (H) and BrdU (I) showing lack of labeling at this invention ages of the large big calbindin-IR V1 cells. Superimposed images of BrdU and YFP (M), BrdU and calbindin (N), and CB and YFP (O). Scale bars; 200 μm in A (B and C are at the same magnification); 100 μm in D (E, F, G, H, and I are at the same magnification); 30 μm in J (K, L, M, N, and O have the same magnification).
Figure 31. BrdU labeling of parvalbumin-IR V1-INs in an En1-Cre/Tau-lacZ animals injected with BrdU at E12.0. A, B, C D, E and F) High magnification images of cells in the Lumbar 2 ventral horn immunolabeled for parvalbumin (white, Cy5, A), nuclear LacZ expression (blue, Alexa-405, B) and BrdU (red, Cy3, D) injected at E12.0. Parvalbumin-immunoreactivity and BrdU are superimposed in E while all three fluorochromes are superimposed in F. In E the solid arrow points to a parvalbumin-IR V1 cell with strong BrdU labeling and the open arrow to the adjacent V1 cell that contains only weak labeling in its nuclei. Parvalbumin-IR V1-derived interneurons have variable cell sizes and locations, most frequently dorsal to the Renshaw cell group. At P15 they represent 8.7% of all V1-derived interneurons at lower lumbar levels and 6.8% at upper lumbar levels. Scale bars; 20 μm in A (all images are at the same magnification).
Figure 32. Percentages of calbindin and parvalbumin immunoreactive V1 interneurons that incorporated BrdU at different ages in En1-Cre/Tau-LacZ animals. A and B) Percentages of calbindin-IR (black bars) and parvalbumin-IR (grey bars) V1 cells that were strongly labeled with BrdU at five different embryonic ages. Lower (A) and upper (B) lumbar segments were analyzed separately. Each bar represents an individual animal. The numbers above each column represent the animal identification code (as in Figure 21). We analyzed three animals at E9.5, E10.5, E11.5, and E12.0 and two animals at E12.5. In each animal 10 ventral horns were counted at each level. In all animals the largest percentage of calbindin-IR V1 cells incorporated BrdU at E9.5, very few incorporated BrdU at E11.5 and E12 and none at E12.5. In contrast, parvalbumin-IR V1 cells are generated from E9.5 to E12.5, but very few incorporate BrdU at E9.5 or E12.5, while peak incorporation occurs at E10.5 and E12.0. C and D) Percentages of cells with strong BrdU labeling in the overall calbindin-IR population (black bars), the Renshaw cell group (light grey), and the big-calbindin-IR V1 cells (dark grey, only in upper lumbar segments). Each average represents the average of three animals, except for E12.5 in which only two animals were analyzed. Error bars represent the S.E.M. Most calbindin-IR V1 cells are generated at E9.5 and E10.5 but there are some generated at E11.5 and E12, however these are not Renshaw cells or big-calbindin-IR V1 cells. Most V1-calbindin-IR neurons generated late were located more dorsally and were not considered Renshaw cells. Renshaw cells and big calbindin V1 cells incorporate BrdU exclusively at E9.5 and E10.5. The percentage of Renshaw cells that incorporated BrdU at E9.5 was significantly higher than at any other age (ANOVA, P<0.05). Differences between E9.5 and E10.5 in the overall calbindin-IR population and the big calbindin cell group did not reach statistical significance. E and F) Pooled animal averages comparing BrdU incorporation in calbindin (black bars) and parvalbumin (grey bars) immunoreactive V1 cells. Same sample size as above. Error bars also indicate S.E.M. At E9.5 the percentage of calbindin-IR V1 cells was much larger than parvalbumin-IR V1 cells in both upper and lower lumbar segments (t-tests, p<0.05). At E11.5 and E12.0 the reverse was true. There were significantly more parvalbumin-IR than calbindin-IR V1 cells with BrdU in both lumbar regions (t-tests, p<0.05). At E10.5 there was no significant difference in BrdU incorporation between V1 cells expressing calbindin or parvalbumin.
Figure 33. BrdU incorporation in Renshaw cells and V1-derived IaINs in En1-Cre/Thy1-YFP P15 mice. A, B, and C) High magnification confocal images of Renshaw cells from lumbar spinal cord sections immunolabeled for YFP (FITC, green, A, B and C), BrdU (Cy3, red, A and C) and calbindin (CB, Cy5, white, B and C) from a P15 animal that was injected with BrdU at E9.5. Many Renshaw cells show strong BrdU labeling at this age. D, E, F, G, H and I) High magnification confocal images of ventral spinal cord sections showing V1-derived IaINs in a P15 animal injected with BrdU at E12.0. D) V1-derived IaINs immunolabeled with YFP (FITC, green). E) BrdU labeling of V1-derived IaINs nuclei. F) Calbindin-IR processes and boutons in the same field. G) Superimposition of YFP and BrdU, showing some V1-derived IaINs strongly labeled for BrdU. H) Superimposition of YFP and calbindin. I) Superimposition of the three fluorochromes. V1 derived IaINs are defines as such because they are innervated by perisomatic baskets of Renshaw cell axons recognized by dual labeling with YFP and calbindin. Scale bars; 20 μm in A and D (B and C are at the same magnification as in A; E, F, G, H, I are at the same magnification as H).
Figure 34. Renshaw cells are born first while V1-derived IaINs are born later in En1-Cre/Thy1-YFP. A and B) Percentage of strongly labeled BrdU cells in Renshaw cells (black bar) and V1-derived IaINs (grey bar) in individual animals. Each bar represents an individual animal coded as in Figure 21. Two animals were analyzed at each BrdU injection time. As before there were no differences between lower (A) and upper (B) lumbar segments and the results were consistent among the two animals and in the two different segmental levels. Also consistent with previous analyses, Renshaw cells in this different independent sample are also labeled by BrdU when injected at E9.5 and E10.5. V1-derived IaINs incorporate BrdU matching the pattern of parvalbumin-IR V1 cells as described in the En1-Cre/Tau-LacZ line (see Figure 32). Almost no cells were labeled with BrdU at E12.5. C and D) Average percentage of strongly labeled cells for both animals showed in histograms A and B. Graphs at both lumbar segments show that Renshaw cells are generated in the first two days (E9.5 and E10.5) while V1-derived IaINs are mostly generated from E10.5 to E12.0.
Renshaw cells are generated at E9.5 and a few at E10.5 (Fig. 34). Almost no V1-IaINs were BrdU-labeled at E9.5 or E12.5. Most IaINs were generated between E10.5 and E12.0, with a maximum at E12 (Fig. 34). The results were consistent in the two animals per age that were used in the quantitative analyses.

In conclusion, V1-derived IaINs are generated later and with a broader generation time frame than Renshaw cells.

**DISCUSSION**

*Methodological considerations of the BrdU birthdating method.*

BrdU was detected using immunohistochemistry in the cell’s nuclei and we distinguished strong and weak nuclear labeling. Strongly labeled cells were interpreted as cells that leave the cell cycle immediately after BrdU incorporation. In this case BrdU is not diluted by further divisions and their nuclei are almost fully covered with strong staining. In contrast, weakly labeled cells appear as spotted labeling within the nuclei and can result from a number of different situations (see explanation in Methods). One possible source of weakly labeled cells is worth discussing further: BrdU might appear spotted because chromatin rearrangements during S-phase. For example at the end of the S-phase the DNA-replication appears punctuated and not uniform and can give raise to speckled staining (Ferreira et al., 1997; Walter et al., 2003) meaning that some cells labeled at the correct time in our study would have been consider weak and discarded. If this is the case we will have underestimated the percentages of cells in S-phase at each injection time, however, this error should be consistent through all ages and animals and overall relationships should be preserved.
Another problem with this technique is the relative low number of cells labeled with BrdU. Injected BrdU is available for only around 2 hours (see Methods). Thus single injections results in relative small percentages of cells labeled. Some authors using \(^3\)H-thymidine overcame this problem by repeated injections after a few hours (Altman and Bayer, 2001). This method resulted in a larger number of labeled cells but with a loss in time resolution. Differences in generation times of different cell types in the spinal cord might be too fast to be well resolved by multiple injections; therefore we chose to use single injections at the expense of labeling just relatively small percentages of V1 cells. Nevertheless, by analyzing large numbers of sections in several lines and animals we were able to recognize very consistent patterns.

A final problem is that with current methods for setting mates and timing pregnancies it is difficult to estimate embryonic stages more accurately than with ±0.5 day of error. Moreover in the embryonic studies described in Aim 2 it was clear that within a single litter different embryos might be at slightly different developmental stages. These uncontrollable variables introduce some error in estimating the exact developmental stage at which BrdU injections were delivered. To minimize errors we used two controls, the overall BrdU pattern in the spinal cord and the thoracic analysis of BrdU-incorporation in cholinergic neurons of known birth-date. Using these controls we are confident that BrdU injections were delivered at the appropriate times of gestation of the animals that were later studied at P15. The problem remains, however, that if the sequence of generation of different types of cells from the p1 domain is faster than ±12 hours, then our methods will not be discriminating enough to detect these differences. In addition, since we expect ±12 hours error estimate, it is possible that
some of the overlap in BrdU incorporation in adjacent dates is due to this error. In this sense it is noteworthy that the studies with different markers were done in the same animals and that for any single marker the study was repeated in different litters and lines. However, it is interesting that the analysis of V1 cells exiting the progenitor area described later in Aim2, suggest a lesser amount of overlap in the production of different cell types than the one we obtained with BrdU labeling.

**Differences in Renshaw cells and IaIN generation from p1 progenitors**

These results support the hypothesis that Renshaw cells and IaINs are generated at different times from the p1 progenitor domain. Renshaw cells are the first cells generated and their time-window for neurogenesis greatly overlaps with that of the motoneurons. These could explain their close relationship. Not only Renshaw cells seem to be generated at early stages, this is also the case for the big-calbindin-IR cells. This could suggest that V1-derived calbindin-IR neurons, in general, exit the cell cycle before other V1-INs (this point is then confirmed in Aim 2). Regarding V1-derived parvalbumin-IR neurons, their peak of generation at E12.0 matches with the one for V1-derived IaINs, suggesting that they are highly overlapping population. In fact, parvalbumin was found expressed in large numbers of V1-derived IaINs at P15 and P20 (Alvarez et al., 2005; Siembab et al., 2010). Overall, Renshaw cells seem to have a much narrower window of generation compared to IaINs. This might suggest that Renshaw cells are a more homogenous population than IaINs. Renshaw cells and IaINs are defined by their function, recurrent and reciprocal inhibition, but whether these functions are mediated by more or less homogeneous subclasses of INs could not be
adequately tested in previous electrophysiological characterizations. In fact, early studies already suggested that reciprocal IaINs could be located at different dorso-ventral regions of LVII, display a variety of sizes and dendritic arborizations, extend axons of different length and orientation and different populations could receive a diversity of inputs from descending systems (reviewed in Jankowska, 1992; Rastad et al., 1990). In contrast, Renshaw cells display a more homogeneous location, morphology and electrophysiological properties (Alvarez and Fyffe, 2007). Recently, our lab revealed, unexpectedly, further diversity among IaINs characterized by histological definition of inputs (Siembab et al., 2010). In this study, IaINs were found to be diverse in regards to their origins from V1’s or non-V1 derived populations and in parvalbumin content (parvalbumin positive and negative populations). Therefore, it is possible that a number of INs with different embryonic development become later incorporated into reciprocal inhibitory circuits. It is possible that if the IaIN populations is composed of different subpopulations and these might be generated at different times.

In conclusion, our studies suggest that different subtypes of V1 INs are generated from p1 progenitors in a temporally regulated manner. This is not always the mechanism followed by ventral INs to generate diversity. For instance, V2-derived INs are generated by asymmetric divisions and through a Notch/Delta lateral signaling pathway that plays a critical role in fating excitatory V2a versus inhibitory V2b INs from the p2 domain. In this case, cell fate is controlled by Delta4 activation of Notch receptors together with MAML factors (Peng et al., 2007). Interestingly, V2a INs display then subpopulations with gradations of function that are generated in a time-
dependent manner. In zebrafish, Alx is the transcription factor analog to Chx10 and defines excitatory V2a INs in the fish (Kimura et al., 2006). Alx V2a INs are glutamatergic INs that correspond with a type of adult INs known as CiD (circumferential ipsilateral descending INs). CiDs are activated during swimming and make monosynaptic excitatory connections with motoneurons (Kimura et al., 2006).

Within this population, there are functional differences according to cell size and this is in turn related to their birth-date. Early born neurons are larger, more dorsally located and active during stronger movements such as escapes or fast swimming, while later born neurons are smaller more ventrally located and are preferentially active during slower and weaker sustained swimming episodes (Kimura et al., 2006). Further analyses demonstrated that small and large CiDs are not recruited by addition with stronger movements but that the INs involved in the weaker movements are silenced during fast movements that recruit INs involved in stronger movements (McLean et al., 2008). This implies not a simple difference according to cell size but also different connectivity for each type of neuron. There are clear differences in our study with this pattern, Renshaw cells are first generated but smaller and more ventrally located than later born V1-IaINs, but nevertheless we wanted to test the possibility of a dorso-ventral gradient of cell generation within the V1 group, overall. Even though we analyzed thoracic segments that seem to preserve a dorso-ventral organization better than in other segments, we could not see a clear dorso-ventral pattern of generation, except that the ventral most cells were generated earlier and the dorsal most V1 cells incorporated BrdU the latest. Thus, we conclude that the dorso-ventral position of mammalian V1 adult INs are related only very weakly to differences in cell generation.
(with the exception of course of the most ventral Renshaw cell group). It could be expected that a larger variety of IN subtypes generated from each progenitor domain in mammals might exhibit a larger variety of migratory patterns, ending in different positions and acquiring different phenotypes. The mechanisms that impose location and connectivity gradients within a single class of INs in zebrafish (CiD) could now be used in mammals to generate distinct subtypes from single progenitors.

Possible mechanism that could regulate V1 phenotype according to time of neurogenesis

The differences in birth-date between Renshaw cells and IaINs within the V1 group suggest two possible mechanisms to define cell type. First their fate could be determined cell autonomously depending on cell cycle number. This mechanism will be akin of generation of neural diversity in Drosophila neuroblasts in which after each cell cycle there is an alteration in transcription factor expression in the progenitor cell altering the fate of daughter cells produced in successive cell cycles (Isshiki et al., 2001). More recently a similar mechanism was discovered for pyramidal cells in the neocortex. As reviewed before corticofugal pyramidal cells located in deep laminae (layer 5 and 6) are generated before pyramidal cells with cortico-cortico projections and located in upper laminae (layer 2 and 3). Transplantation studies using heterochronic or isochronic cells found that the developmental potential of cells becomes narrower with time. For example, early progenitors, which normally produce deep pyramidal cells, will generate superficial laminae cells if transplanted in an older cortex, but late progenitors will generate layer2/3 pyramidal cells even if transplanted to an earlier
environment (McConnell and Kaznowski, 1991). More recent studies based on clonal analyses suggested that the progenitors sequentially generate lower and upper pyramidal cells by restricting their fate potential over time (Shen et al., 2006). These changes are accompanied by changes in transcription factor expression in the progenitors and even translocation of the progenitors from the ventricular to the subventricular zone (reviewed by Leone et al., 2008).

Are there similar mechanism operating in the p1 domain? At present this is unclear. First, our data cannot address the question of whether early and late generated V1 INs are clonally related or not. This means whether they arise from different cell cycles of the same progenitor or by different progenitor subdomains within the p1 domain. Second, we do not know if the sequence we observed is due to a restriction of progenitor potential with cell cycle divisions or to complete different cell type specifications. It is however, intriguing that some regions in the p1 domain downregulate expression of the transcription factor Dbx1 after E10 (Pierani et al., 2001), that is after Renshaw cells have been generated. Levels of Sonic hedgehog and retinoic acid vary with development (Ericson et al., 1995; Ulloa and Briscoe, 2007; Maden, 2001) and these changes could modify transcription factor expression at the level of the ventral progenitors as it occurs when the pMN domain switches form generating motoneurons to oligodendrocytes (reviewed in background). These mechanisms are not necessarily exclusive since changes in genetic determination established by a time-clock at the progenitor level could determine competences to respond to temporally regulated environmental influences in the differentiating neurons.
Future experiments should address these more specific issues but before that we need to know first at what time these cells start differentiation. Our birth-dating study does not provide information on the exact timing of differentiation for each of these two cell populations; however, given their different birth dates it is possible that these cells start to differentiate just after they are born. If this is true, they should differentially regulate gene expression at a very early stage and this should be reflected in the expression of different markers. Alternatively, V1 cells generated at different times could remain relatively undifferentiated until the expression of different cues later in development. In aim 2 we will test the hypothesis that there is an early differentiation and that Renshaw cells and IaINs can be distinguished in early embryos within the V1 population by their expression of different transcription factors or phenotypic markers.
AIM 2

CHARACTERIZATION OF TRANSCRIPTION FACTOR EXPRESSION IN RENSHAW CELLS AND V1-DERIVED IA INHIBITORY INTERNEURONS IN THE EMBRYONIC AND POSTNATAL SPINAL CORD
INTRODUCTION

The results in aim 1 suggest that Renshaw cells and V1-derived IaINs are generated sequentially and therefore start development at different embryonic times. Their different birth dates could imply that differentiation from each other begins early, perhaps just at the time they exit from the progenitor cell cycle. If this was the case they might be fated by their differential birth date and each cell type would follow different mechanisms of differentiation and integration into the ventral horn spinal cord circuits. An alternative possibility is that although their time of generation is different, they could remain as undifferentiated postmitotic cells in the early embryonic spinal cord and split into Renshaw cell and IaIN phenotypes at later times, for example at the time of early synaptogenesis, which in the mouse embryo occurs between E12 and E13 (Vaughn et al., 1975). In this case the early embryonic cord could contain precursors of Renshaw cells and IaINs with similar properties and functionality until the start of spinal circuit assembly.

Analyses of the diversification of other ventral spinal cord neurons suggest that an early specification is the more likely possibility, although later signals might add further phenotypic complexity or even impose switches in development, for example from one neurotransmitter to another (reviewed in Edlund and Jessell, 1999). In motoneurons, specific transcription factor expression is regulated hierarchically in a temporal manner such that motoneurons express them in different combinations while they become sequentially restricted into different classes, columns, divisions and pools (reviewed in Jessell, 2000; Price and Briscoe, 2004; Poh et al., 2002; Ji et al., 2009).
Transcriptional codes that restrict the class, columnar and divisional identity of motoneurons occur very early in development, while ETS transcription factors that define pool identity are expressed later, simultaneously with muscle innervation and are in part controlled by the periphery (Jessell, 2000; Price and Briscoe, 2004). It is possible that IN lineages undergo a similar diversification process starting just at the time that the progenitor becomes postmitotic. One such example is the division of the p2 progeny into excitatory V2a INs that express the transcription factor Chx10 and V2b INs that express GATA3 (Peng et al., 2007). V2a and V2b INs develop simultaneously from genetically identical p2 progenitors through a lateral delta4-Notch signaling mechanism that produces daughter cells of different phenotypes. Early differentiation would also agree with the existence of discrete groups of INs expressing unique combinations of transcription factors in the very early spinal cord. One such example are the cholinergic INs that form partition cells (PC) in the mature spinal cord and give raise to C-terminal synapses on the motoneurons. These cells arise from a subgroup of V0 INs that expresses the transcription factor pitx2 from the early embryo (E12) to adult (Zagoraiou et al., 2009). An early differentiation of V1 INs giving rise to Renshaw cells or V1-derived IaINs would indicate that they belong to different branches within a possible hierarchical organization for the specification of V1 subtypes. Since transcription factors have the intrinsic ability to translate transient extrinsic signals into long-lived cellular responses and early differentiation is almost always reflected in the expression of different transcription factors we searched for potential transcription factors that could define subgroups within the V1 population.
In a previous preliminary study it was suggested that subpopulations of embryonic V1-INs could be differentiated based on the expression of two transcription factors, MafB and FoxP2 (Geiman et al., 2007). MafB expressing V1 INs were described in the Renshaw cell area and FoxP2 expressing V1 INs were more dorsally located, in a region where we might expect to find developing IaIN precursors. Therefore we analyzed whether these transcription factors divide the V1 population in early born Renshaw cells and late born V1-derived IaINs and at what stage they are first expressed in the embryonic spinal cord.

MafB belongs to the MAF family of genes that are widely expressed at varying levels and in distinct spatiotemporal patterns. The MAF proteins are important during neurogenesis and exert transcriptional control over gene expression, development and differentiation. The role of MAF proteins in development and differentiation and their importance in neurogenesis makes MafB a good candidate to be involved in the control of V1 differentiation (Wang et al., 1999). FoxP2 is a transcription factor that belongs to a family of genes named FOX that are conserved from fungi to mammals and that have been associated in humans with articulation of complex speech sounds, since mutations of this gene are associated with language deficits (Fisher and Scharff, 2009). FoxP2 is also involved in various early embryogenesis processes, including neural differentiation (Bonkowsky and Chien, 2005; French et al., 2007). Furthermore, FoxP2 is expressed in a subpopulation of V1-INs in mouse embryos. FoxP2 was detected from E11.5 to P0 mostly in the ventral spinal cord (Morikawa et al., 2009), suggesting very early expression.
We hypothesized that MafB and FoxP2 could be early markers of Renshaw cells and IaINs, respectively. Furthermore, we hypothesized that since Renshaw cells are among the first V1 INs generated, expression of the MafB transcription factor in V1-INs should precede expression of FoxP2 in the embryonic spinal cord and within the V1 population. To test these ideas we first compared the expression of MafB and FoxP2 in characterized subpopulations of V1-INs in the postnatal spinal cord. Given the fast downregulation of these transcription factors after birth the studies were constrained to P0 and P5. Then, we analyzed their distribution and onset of expression in the embryonic spinal cord.

MATERIALS AND METHODS

Animals

We used for postnatal analyses En1-Cre/Thy1-YFP and En1-Cre/Tau-LacZ animals. However we noted that in these animals upregulation of reporter expression occurs late during embryogenesis in the V1 population. In particular Renshaw cell are among the last cell populations to express these reporters within V1 INs. Therefore, for embryo studies we crossed En1Cre/+ heterozygotes with the CAG-Rosa26-lox-STOP-loxp-tdTomato-WPRE reporter line (stock#007909; Jackson labs, Bar Harbor, Maine). We will refer to these animals as En1-Cre/R26-tdTomato. Initially we purchased this line as heterozygotes and therefore only 25% of the litter expressed tdTomato in V1 cells. All transgenic animals were bred at Wright State University. Pups were tail clipped for genotyping using Polymerase Chain Reaction (PCR) and the feet tattooed
before P5 for identification as before. All animal procedures were performed according to NIH guidelines and reviewed by the local Laboratory Animal Use Committee at Wright State University.

**Tissue extraction**

Fourteen *En1-Cre/Thy1-YFP* or *En1-Cre/Tau-LacZ* mice pups were anesthetized at either P0 or P5 with Euthasol (2.0 μg/g i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer (PB). After perfusion the spinal cords were dissected and postfixed overnight in the same fixative and then cryoprotected in 0.1M PB (pH 7.4) with 30% sucrose and 0.01% sodium azide. The spinal cords were stored at 4°C in this solution until used.

In addition, 12 timed pregnant females were used to generate litters containing *En1-Cre/Tau-LacZ* (4 litters) and *En1-Cre/R26-tdTomato* (8 litters). At the appropriate gestation times (E.95 to E12.5) the pregnant females were anesthetized and perfused as above and the embryos removed from the uterus and freed from their yolk sacs. Embryos were fixed *in toto* overnight and then cryoprotected in 30% sucrose. Embryonic developmental stages were confirmed using the Atlas of Mouse Development (Kaufman, 2005). Embryos were cut in a cryostat at 20-30 μm thickness and collected in subbed slides. Slides were stored at -20°C until processed. In addition we analyzed in a preliminary study a few slides containing sections from *En1-Cre/Thy1-YFP* and *En1-Cre/Tau-LacZ* late embryos (E15.5, E17.5). These were provided by Dr. Valerie Siembab in our lab.
**Expression of transcription factors in V1-INS postnatally**

Six *En1-Cre/Thy1-YFP* animals at P5 and five at P0, as well as three *En1-Cre/Tau-LacZ* P0 animals, were used for postnatal analysis of transcription factor expression in V1-derived Renshaw cells and IaINs. Sections were triple or quadruple immunolabeled for either polyclonal sheep antibody against GFP (1:800, Biogenesis, Brentwood, NH) or chicken polyclonal antibodies against β-gal (1:500, AbCam Inc., Cambridge, MA) combined with polyclonal rabbit antibodies against calbindin (1:500, Swant, Bellizona, Switzerland), polyclonal guinea pig antibodies against VGLUT1 (1:5000, Synaptic Systems, Goettingen, Germany), and goat polyclonal antibodies against either MafB or FoxP2 (1:200, Santa Cruz biotechnology Inc., Santa Cruz, CA). Immunoreactive sites were revealed with fluorochrome-conjugated (FITC, Cy3, and Cy5) donkey species-specific secondary antibodies (1:50, Jackson Laboratories) or Alexa-405 (Invitrogen) as previously described and all immunofluorescent preparations were coverslipped with Vectashield (Vector Labs).

**Analysis**

Images were obtained in an Olympus FV1000 confocal microscope at 20x1 and 60x1. Images were analyzed and labeled cells identified counted using Fluoview software (Olympus). Six P5 *En1-Cre/Thy1-YFP* animals were analyzed for FoxP2 expression and 6 P0 *En1-Cre/Thy1-YFP* and 3 P0 *En1-Cre/Tau-LacZ* animals were studied to characterize MafB expression. Ten ventral horns per animal were sampled.
The number of MafB and FoxP2 immunoreactive cells within the V1, Renshaw cell and IaIN populations were calculated. Renshaw cells were identified due to their location and calbindin-immunoreactivity as in Aim 1. V1-derived IaINs were identified as V1-INs receiving convergent inputs from both Renshaw cells (YFP V1 labeled calbindin-IR axonal contacts) and vesicular glutamate transporter 1 (VGLUT1) contacts from sensory proprioceptors. We obtained percentages of expression in six En1-Cre/Thyl-YFP animals for the FoxP2 study. Initially we also analyzed P0 En1-Cre/Thyl-YFP animals for MafB expression but we noted that a large number of Renshaw cells did not contain YFP at this age. Because of this reason the MafB analysis was repeated in three En1-Cre/Tau-LacZ animals at P0. All calbindin-IR Renshaw cells express the LacZ reporter at P0 in this line.

Expression of transcription factors during development at different embryonic ages

In preliminary studies we dual immunostained late embryos (E15.5 and E17.5) of the En1-Cre/Thyl-YFP and En1-Cre/Tau-LacZ for the reporter and calbindin (as above) and found that at this age reporter expression does not occur in the En1-Cre/Thyl-YFP line. We focused then in the En1-Cre/Tau-LacZ line.

A total of 9 En1-Cre/Tau-LacZ embryos were used at three embryonic stages (E10.5, n = 2, from one litter; E11.5, n = 2, from one litter; E12.5, n = 5, from two litters) to analyze the developmental expression of MafB and FoxP2 in V1-INs. Sections were triple immunolabeled with chicken polyclonal antibodies against β-gal (1:500, AbCam Inc., Cambridge, MA) combined with rabbit polyclonal antibodies against calbindin (1:500, Swant, Bellizona, Switzerland), and MafB or FoxP2 goat
polyclonal antibodies (1:200, Santa Cruz biotechnology Inc., Santa Cruz, CA). Immunoreactive sites were revealed with fluorochrome-conjugated (FITC, Cy3, and Cy5) donkey species-specific secondary antibodies (1:50, Jackson Laboratories) and cover slipped as previously described. In these animals we noted that putative calbindin-IR Renshaw cell precursors did not express LacZ at E10.5 and that the reporter was first observed in some Renshaw cells only by E11.5. As above this suggests late upregulation of Tau promoter activity driving reporter expression in embryonic Renshaw cells. Therefore we repeated the experiments in En1-Cre/R26-tdTomato embryos at E9.5 (n = 3, from one litter), E10.5 (n = 4, from two litters), E11.5 (n = 7, from two litters), E12 (n = 4, from two litters) and E12.5 (n = 3, from one litter). In these animals tdTomato expression was visualized “naked” (without immunocytochemical amplification) and the sections were dual immunolabeled for calbindin (1:500, rabbit polyclonal antibody, Swant) combined with either MafB or FoxP2 (1:200; goat polyclonal antibodies, Santa Cruz Biotechnology Inc).

Images were obtained with an Olympus FV1000 confocal microscope at 20x1 and 60x1. Unfortunately, even at 20 µm thickness and using confocal microscopy the cellular density in these early embryos is too dense for reliable counting. Therefore the materials are described qualitatively. Image composition was performed as in aim 1.
### Table 3. Antibodies used in Aim 2 and labeling specificity

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Type</th>
<th>Host-species</th>
<th>Dilution</th>
<th>Company</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
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<td>Calbindin D28K</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Swant, Bellinzona</td>
<td>No labeling in KO tissue</td>
</tr>
<tr>
<td>VGLUT1</td>
<td>Polyclonal</td>
<td>Guinea pig</td>
<td>1:5000</td>
<td>Synaptic Systems</td>
<td>No labeling in KO</td>
</tr>
<tr>
<td>β-gal</td>
<td>Polyclonal</td>
<td>Chicken</td>
<td>1:1000</td>
<td>Abcam Inc.</td>
<td>No labeling in animals with no reporter expression</td>
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<tr>
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<td>Chicken</td>
<td>1:5000</td>
<td>Aves Labs</td>
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<tr>
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<td>1:200</td>
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<tr>
<td>FoxP2</td>
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<td>1:200</td>
<td>Santa Cruz Biotech.</td>
<td>Per Santa Cruz data sheet</td>
</tr>
<tr>
<td>NeuN</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Chemicon</td>
<td>Only antibody available (ref. Kim et al., 2009)</td>
</tr>
</tbody>
</table>

### RESULTS

MafB and FoxP2 were found in the nuclei of many V1 and non V1-INs in the postnatal spinal cord (Fig. 35). In preliminary analyses carried out in our lab by Mrs. Maria Berrocal it was found that MafB was quickly downregulated after P0 and immunolabelings became inconsistent by P5. In contrast, FoxP2 expression was similar at P0 and P5 and downregulated more gradually. By P10 fewer cells were found and by P15 there were very few FoxP2 positive neurons in the ventral spinal cord and these were more weakly labeled (not shown). The studies described here in the postnatal spinal cord therefore focused at P0 (MafB) and P5 (FoxP2).
Figure 35. Distribution and expression of MafB and FoxP2 in V1-derived interneurons of respectively, P0 En1-Cre/Tau-lacZ and P5 En1-Cre/Thy1-YFP spinal cords. A to C) Low magnification confocal images of the lumbar spinal cord from an En1-Cre/Tau-lacZ animal at P0 immunostained for MafB (Cy3, red, A-C), LacZ (FITC, green, B) and calbindin (Cy5, white, C). A) In the P0 spinal cord MafB is expressed in a ventrally located group located in the Renshaw cell area, a more lateral group of cells in lamina IX and several dorsal populations. B) The most ventral group of MafB-immunoreactive cells belong to the V1 group (LacZ positive). C) Merge of calbindin and MafB immunoreactivities demonstrates that this ventral group corresponds with Renshaw cells. D, E and F) High magnification confocal images of Renshaw cells (arrows) immunostained for MafB (Cy3, red), LacZ (FITC, green), and calbindin (Cy5, white). G to I) Low magnification confocal images of lumbar spinal cord sections at P5 immunolabeled with FoxP2 (Alexa-405, blue, G, H, and I), YFP (FITC, green, H), and calbindin (CB, Cy5, white, I). G) Distribution of FoxP2 in P5 lumbar segments. FoxP2-immunoreactive cells are for the most part localized in the ventral horn. H) Some V1-INs (YFP positive) express FoxP2. I) FoxP2 is not expressed by calbindin-IR V1 Renshaw cells. J, K and L) High magnification confocal images of an YFP (FITC, green) V1-IN immunostained for FoxP2 (Alexa-405, blue, J). This cell receives contacts from Renshaw cell axons that are both calbindin-immunoreactive (Cy5, white) and YFP positive (K). It also receives contacts from VGLUT1 boutons (Cy3, red, white arrows in L). This particular cell is therefore a V1-derived IaIN. M and N) Percentages of cells of different groups of V1-INs with either MafB of FoxP2 in the En1-Cre/Tau-LacZ and En1-Cre/Thy1-YFP lines; 100% of V1 Renshaw cells express MafB, while less than 10% of V1-INs express MafB. Six animals were analyzed in the En1-Cre/Tau-lacZ line and three in the En1-cre/Thy1-YFP. In contrast, around 70% of V1-derived IaINs express FoxP2 at P5 and 33% of V1 overall express FoxP2. Scale bars; 200 µm in A and G (images B, C, H and I are at the same magnification).
**MafB expression in V1-interneurons in at P0**

Analysis of P0 spinal cords in *En1-Cre/Thy1-YFP* and *En1-Cre/Tau-LacZ* spinal cords (Fig. 35A-F, M) proved that 100% of V1-derived calbindin-IR Renshaw cells located in ventral lamina VII express MafB. However, YFP expression in P0 Renshaw cells in the *En1-Cre/Thy1-YFP* was variable. On average, 17 ±6% of calbindin-IR neurons in the Renshaw cell area were YFP negative at P0 (n = 6 animals). Positive reporter expression varied in different animals from 98% of calbindin-IR Renshaw cells to 62%. In contrast, there is 100% positive expression of genetic markers in postnatal Renshaw cells when using a R26 reporter line (Sapir et al., 2004) or the *En1-Cre/Tau-LacZ* line (Fig. 36E-H). At P5 and later ages (Aim 1) all Renshaw cells consistently express YFP in the Thy1 line (Fig. 36M-P). This result suggests late upregulation of YFP expression in Renshaw cells during postnatal development.

Renshaw cells were not the only MafB expressing cell group in the spinal cord (Fig. 35A-C). A dorsal horn group, unrelated to V1-INs also expressed mafB. These cells were located throughout LIII, IV and particularly in lateral LV. More interestingly a group of dorsal V1 INs (located in dorsal regions of LVII), that differs from Renshaw cells and do not express calbindin, also expresses MafB. Quantitatively the dorsal V1 group was much smaller than the Renshaw cell group. Overall around 10% of V1-INs expressed MafB. MafB-immunoreactivity was strongly downregulated after P0.

In conclusion, MafB expression is not unique to Renshaw cells, but all Renshaw cells express this transcription factor at P0.
Figure 36. Distribution and expression of reporters in V1-derived interneurons in En1-Cre/Tau-lacZ and En1-Cre/Thy1-YFP animals during development. A-H) Low magnification confocal images of spinal cords from E11.5 to P15 showing the distribution of V1-INs in En1-Cre/Tau-lacZ animal. I to P) Similar confocal images but in the En1-Cre/Thy1-YFP animals. V1-INs are shown in green (FITC, lacZ positive in Tau-LacZ and YFP positive in Thy1-YFP). Calbindin-IR is shown in white (Cy5). Expression of the LacZ reporter is first detected at E11.5 (A), however it is not very strong and quite diffuse and is difficult to individualize to specific cell types, especially the calbindin-IR cells (B). By E12.5 (C) LacZ expression is more defined in the nuclei of the cells and brighter. However, not all V1-INs express the reporter at this age. Specifically, upregulation of LacZ expression in Renshaw cells is delayed compared to other V1-INs. LacZ is not detectable in a large number of calbindin-IR cells located in the Renshaw cell area (C,D). By P0 all calbindin-IR Renshaw cells express the reporter (E,F), but there many other calbindin-IR interneurons in the ventral horn. At P15 calbindin-immunoreactivity has become restricted to Renshaw cells in the ventral horn and all Renshaw cells express lacZ (G,H). In contrast, YFP expression is not detected in V1-INs until E15.5 (I) and it is extremely weak at this point. YFP expression becomes stronger by E17.5 (K), but it is still not present in calbindin-IR Renshaw cells. By P5 (M), YFP seems to be upregulated in 75% of all V1-INs, including all Renshaw cells by P5 (M,N). YFP expression is maintained in all calbindin-IR Renshaw cells at P15 (O, P). These results indicate that upregulation of both reporters in Renshaw cells happens later than in the rest of V1-INs. Scale bars; 100 µm in A (all images are at the same magnification).
FoxP2 expression in V1-interneurons in the postnatal spinal cord

These analyses were performed at P5 in En1-Cre/Thy1-YFP spinal cords to optimize identification of V1-derived IaINs (Fig. 35G-L, N). Convergent inputs from Renshaw cells (axons co-labeled with YFP and calbindin) and proprioceptive afferents (VGLUT1 immunoreactive) were used as criteria for IaIN identification. Analyses performed at P5 allowed clearer identification of primary afferent inputs given that VGLUT1-immunoreactivity inside the central terminals of primary afferents undergo considerable upregulation during postnatal development (Mentis et al., 2006; Siembab et al., 2010). Also by P5 calbindin immunoreactivity has downregulated in most non-Renshaw V1-INs in lower lumbar regions (Siembab et al., 2010), becoming a more specific marker of Renshaw axons.

FoxP2 is more widely expressed in the postnatal spinal cord ventral horns than MafB (Fig. 35G). Fewer neurons express FoxP2 in the dorsal horn. In the P5 ventral horn FoxP2 is present in several groups of V1 and non V1-derived INs (Fig. 35G-I) and in cells that can be identified as V1-derived IaINs (Fig. 35J-L). FoxP2 cells are a relatively large population and 40% were identified as V1-derived IaINs (Fig. 35N). Thirty-three percent of YFP V1-INs expressed FoxP2 and around 66% of V1-derived IaINs expressed FoxP2. FoxP2-IaINs were a relatively small population (11%) of all V1-derived INs expressing FoxP2 and were a small percentage (5%) of all ventral horn FoxP2 cells. Therefore we conclude that several classes of V1-INs in addition to IaINs express FoxP2. FoxP2 was never expressed by Renshaw cells. Therefore, MafB and FoxP2 are expressed in distinct subgroups of cells within V1-derived INs.
In conclusion, MafB and FoxP2 cannot be regarded as specific of Renshaw cells and IaINs, respectively, but they can be used as markers to label within the V1 population all Renshaw cells and a large number of IaINs. We therefore used these markers to analyze the divergence of Renshaw cells from IaINs in the early embryonic spinal cord.

*Location and morphology of V1-interneurons in early embryos.*

We first analyzed *En1-Cre/Tau-LacZ* embryos at E10.5, E11.5 and E12.5, but we were unable to detect any labeling of V1 cells in these embryos at E10.5 (not shown). Labeling was weak at E11.5 and increased by E12.5 (Figs. 36A-D). Many putative V1 calbindin-IR Renshaw cells (identified based on location) lacked LacZ, particularly at E11.5. LacZ expression is robust in Renshaw cells at P0 and P15 (Fig. 36E-H). To confirm whether lack of labeling was due to developmental regulation of reporter expression in the Tau line or because late upregulation of En1 in some V1 INs, we repeated the experiments in *En1-Cre/R26-tdTomato* mice. In these animals no V1-cells were found at E9.5 (Fig. 37A, B) but we found labeled cells at E10.5 (Fig. 38A). Given the ubiquitous expression of the CAG reporter and its location in the Rosa26 locus, which allows transcription from very early stages in the embryo, it is unlikely that lack of reporter expression in the E9.5 spinal cord is due to inactivity of the promoter. Indeed, tdTomato labeling is clearly visible in dorsal midbrain progenitors known to express En1 by E9.5 (Fig. 37C, D). The differences in timing between birth dates (Aim 1) and genetic labeling of embryonic V1-INs are therefore likely related to
Figure 37. Lack of R26-tdTomato reporter and calbindin expression in the spinal cord of E9.5 embryos. A to D) Low magnification confocal images spinal cord (A and B) and midbrain (C and D) cross-section in E9.5 embryos. tdTomato expression (V1, in red) is not visible in the spinal cord but it is strongly expressed by dorsal progenitors in the neural tube at midbrain regions. E to F) Calbindin-immunoreactivity (CB in green) is undetectable in the same fields shown in A-D of spinal cord and midbrain. Scale bars; 100 µm in A, B, C and D (matching images in E, F, G and H are at the same magnification). The early neural tube at the level of the spinal cord is outlined in A, B, E and F.
Figure 38. Location of V1-INs and calbindin-immunoreactivity from E10.5 to E12.5 in the En1-Cre/R26-tdTomato mouse model. Solid lines indicate the boundaries of the early spinal cord. Dotted lines indicate the ventricle in the midline of the spinal cord. All images are low magnification confocal images immunostained for calbindin (Cy5, white, B, E, H, and K) and showing genetically labeled V1-INs (tdTomato, red, A, D, G, J). A to C) E10.5 spinal cord contains a small cluster of V1-INs leaving the progenitor area and sending ventral projections (A). Most of these V1-INs express calbindin-immunoreactivity (B,C). D to F) At E11.5 the numbers of V1-INs (D) and calbindin-IR cells (E) increased. Most cells located laterally express both markers (F) and are positioned either at the edge of the ventro-lateral spinal cord (arrows in E) or medial to the motor pool. Another group of more weakly labeled V1-INs is at border of the progenitor area. Many of these cells do no express calbindin and appear to be migrating away from the progenitor area. G to I) V1-INs at E12 are still leaving the progenitor area. The most lateral clusters of calbindin-IR V1 interneurons start to form a Renshaw cell group, while medial V1-INs are either calbindin positive or negative. At this age many V1-INs are seen exiting the progenitor zone and all of these are without exception calbindin negative. J to L) At E12.5 no V1-INs are exiting the progenitor area and the number of calbindin negative V1 interneurons in the ventral horn has significantly increased. Scale bars; 100μm in all images.
the time necessary for cells to transfer from S-phase to cell division and then start of V1-differentiation and upregulation of En1 (see discussion).

V1-INs detected at E10.5 are restricted to a very small group of cells located in a single mid dorso-ventral plane and in between the ventricular zone and the lateral external border of the spinal cord (Figs 38A, 39A, 40A, 44A). The more lateral cells extend several neurites while the more medial cells are frequently bipolar and express weaker tdTomato labeling. As previously suggested in motoneurons (Wentworth, 1984), this morphological changes might represent a medial to lateral progression in maturation, being the morphology of lateral cells more mature than medial ones. Medial cells are usually located in a transition zone between the ventricular progenitor zone and the mantle layer. This space is small at this age and these cells seem to be the first to occupy and form the mantle region at this dorso-ventral location. The number of V1-INs increased at E11.5 and their area of distribution extended more ventrally occupying positions both lateral and medial to presumptive motor pools in the ventral horn (Figs. 38D, 40A, 44A). Further V1-INs are added at E12 and E12.5, mostly medial to putative motor pools. The ventral horn grows in size by the addition of more cells (Figs. 38G,J, 41A, 42A, 45A, 46A). At E11.5 and E12 many small V1-INs are located medially, at the border of the ventricular zone. These cells form tight packets of high cellular density and weak tdTomato labeling and likely represent newborn cells. In our preparations is usually difficult to discern single cells within these packets. The morphological complexity of V1-INs and tdTomato expression both increased in the more lateral cells suggesting they have further progressed in their development. Higher tdTomato expression permitted better cell definition in the lateral cells. At E12.5 we
Figure 39. Expression of MafB in V1-INs in the early embryonic spinal cord (E10.5). Low and high magnification confocal images of spinal cord sections obtained from an En1-Cre/R26-tdTomato mouse. The solid line delineates the embryonic spinal cord, while the dashed line marks the midline. The dotted line in G to I indicates the edge of the progenitor area. V1-INs are labeled in red (tdTomato), MafB-immunoreactivity in green (FITC) and calbindin-IR in white (Cy5). A to F) Few V1-INs have been generated at E10.5 (A) and the few that are present are located in between the progenitor area and the external border of the spinal cord. At this age, MafB expression is mostly present in motoneurons (B) and not in V1-INs (E, F, H and I). However, calbindin-IR (C) is present in most V1-INs at this age. G to I) Higher magnification images with the boundary between progenitor area and mantle layer indicated (dotted line). Calbindin-IR V1-INs are located either laterally (cells sending projections ventrally) or medially. The medial cells are bipolar and seem to be migrating away from the progenitor area. None of these cells express MafB. Scale bar; 100 μm in A (panels B to F at the same magnification); 50 μm in G (panels H to I are at the same magnification).
**Figure 40. Expression of MafB in the E11.5 spinal cord.** Low magnification confocal images of the spinal cord from an *En1-Cre/R26-tdTomato* mouse. Markings as in Figure 39. Sections were immunostained for MafB in green (FITC) and calbindin in white (Cy5). V1-INs are labeled red (tdTomato). At E11.5 there appears to be considerably more labeled V1-INs than at E10.5 (red, A). A group of V1-INs are already ventrally located. MafB expression has been downregulated from motoneurons (B) and it starts to be present in few V1-INs. Calbindin-IR is mostly located ventrally (C and D) in the majority of laterally located V1-INs. Scale bar; 100 µm in A and applies to all images.
Figure 41. Expression of MafB in V1-INs in the E12.0 embryonic spinal cord. Low magnification confocal images of the spinal cord from an En1-Cre/R26-tdTomato mouse. Markings as in Figure 39. Sections were immunostained for MafB (green, FITC) and calbindin (white, Cy5). V1-INs are visualized naked (red, tdTomato). V1-INs can still be seen exiting the progenitor area (A) and some of the more ventrally located now express MafB (B, E and F) and calbindin (C, D and E). Calbindin expression is, at this age, located in the most ventral areas of the ventral horn (C), while MafB expression has been almost completely downregulated from motoneuron pools and it is present in V1-INs, mostly in the ones most ventrally situated. Scale bar; 100 µm in A and is applicable to all images.
Figure 42. Expression of MafB in V1-INs in the E12.5 embryonic spinal cord. Low magnification confocal images of the ventral spinal cord from En1-Cre/R26-tdTomato. Markings as in Figure 39. Sections were immunostained for MafB (green, FITC) and calbindin (white, Cy5). V1-INs are visualized naked (red). By E12.5 no more V1-INs can be seen exiting the progenitor area (A) and most cells appear to be located in their final positions with regard to the motor pools. MafB (B) is present in the more ventrally located V1-INs that also express calbindin (C). These cells are likely developing Renshaw cells. Bottom row shows superimpositions of two fluorochromes. Scale bar; 100 µm in A, applies to all images.
could not detect cells exiting from the ventricular zone and the majority of V1-INs appear relatively mature.

**Calbindin expression in early embryos.**

From E10.5 to E12.5 a proportion of V1-INs expressed calbindin-immunoreactivity (Fig. 38). No calbindin-IR cells were detected at E9.5 in the spinal cord or midbrain (Fig. 37E-H), therefore the first calbindin-IR cells detected in the spinal cord are V1-INs. The majority of V1-INs at E10.5 and most of the laterally located and more mature V1-INs at E11.5 are calbindin-IR (Figs 38A,D, 39A,D, 40A,D, 43A,D, 44A,D). Calbindin seems to upregulate in these cells very early, being frequently found in cells exiting the ventricular zone at E10.5 (Fig. 39). By E12 and E12.5 a large number of the newly added V1-INs are calbindin negative and newborn cells exiting the ventricular zone at E11.5 and E12 are also calbindin negative (Figs. 41A,D, 42A,D, 45A,D, 46A,D). Presumptive calbindin-IR V1-Renshaw cells are located between motoneurons and the lateral edge of the spinal cord by E11.5, and these cells remain more or less at a similar position at E12 and E12.5 (Fig. 38). In conclusion, the first wave of V1-IN neurogenesis seem to correspond with cells that rapidly upregulate calbindin in agreement with the conclusions from aim 1.

In addition, we found populations of calbindin-IR cells that are not V1-INs at E12 and E12.5. Some are relatively large and could represent motoneurons based on the fact that some calbindin-IR axons can be seen exiting the spinal cord though the ventral roots. Motoneurons are known to downregulate calbindin expression by P0 (Zhang et al., 1990).
Expression of transcription factors in embryonic V1-interneurons

MafB expression in the embryonic spinal is detected prior to FoxP2, but surprisingly most was found in motoneurons and not in V1-INs at E10.5 (Figs. 39). By E11.5 MafB expression downregulates in motoneurons and starts to be upregulated in some V1-INs (Fig. 40). At this age MafB expression is found in calbindin-IR V1-INs that start to be located at the edge of the spinal cord lateral to motoneurons and also in some dorsally located V1-INs some of which are calbindin negative. Not until E12.0, could we detect MafB strongly expressed in most calbindin-IR V1-INs in the Renshaw region (Fig. 41) and this expression is maintained at E12.5 (Fig. 42) and until P0 (see above). In conclusion, MafB expression in Renshaw cells is delayed compared to calbindin expression and upregulates only after the cells have reached their final locations. MafB is therefore upregulated at a relatively advanced step in differentiation in two groups of V1-INs (ventral Renshaw cells and dorsal non-calbindin V1-INs). Both seem generated earlier than E11.5.

In contrast, there are no FoxP2 cells at E10.5 (Fig. 43). The first expression is observed at E11.5 in a group of packed V1-INs located at the exit region of the progenitor zone (Fig. 44). Similar newborn V1-INs expressing FoxP2 are found at E12, but not at E12.5 (Figs. 45 and 46). E12 and E12.5 are characterized by the increase in the number of FoxP2 expressing V1-INs that are located in the marginal region (Figs. 45 and 46). In conclusion, FoxP2 expression seems characteristic of late born V1-INs but is upregulated in these cells as soon as they start differentiation.
Figure 43. Expression of FoxP2 in V1-INs in the E10.5 spinal cord. Low magnification confocal images of a spinal cord from an En1-Cre/R26-tdTomato mouse. Markings as in Figure 39. Sections were immunostained for FoxP2 in green (FITC), and calbindin in white (Cy5). V1-INs are visualized in red (tdTomato). The locations of V1-INs and calbindin immunoreactivity is as previously described (Figure 39). No FoxP2 immunoreactivity is visible at this age. Scale bar; 100 μm in A, applies all images.
Figure 44. Expression of FoxP2 in V1-INs in the E11.5 spinal cord. Low magnification confocal images of spinal cords from an En1-Cre/R26-tdTomato mouse. Markings as in Figure 39. Sections were immunostained for FoxP2 in green (FITC), and calbindin in white (Cy5). V1-INs are visualized naked in red (tdTomato). At E11.5 we can first detect FoxP2 (B) expression in some V1-INs (A) that are just exiting the progenitor area. None of these cells express calbindin-immunoreactivity (C). D to F superimposition of different fluorochromes. Scale bar; 100μm in all images.
Figure 45. Expression of FoxP2 in V1-INs in the E12.0 spinal cord. Low magnification confocal images of spinal cords from an En1-Cre/R26-tdTomato mouse. Markings as explained in Figure 39. Sections were immunostained for FoxP2 in green (FITC), and calbindin in white (Cy5). V1-INs are visualized naked in red (tdTomato). At E12.0 we detect many V1-INs (A) leaving the progenitor area and these cells also express FoxP2 (B and F). In addition, many V1-INs that are FoxP2 positive have already migrated ventrally and display more complex morphologies. Calbindin-IR (C) at this age is mostly restricted to ventral locations in the spinal cord and colocalizes with the most ventral V1-INs (D), but none of this are FoxP2-immunoreactive (E). Scale bar; 100 μm in A, applies to all images.
Figure 46. Expression of FoxP2 in V1-INs in the E12.5 spinal cord. Low magnification confocal images of a ventral spinal cord from an En1-Cre/R26-tdTomato mouse. Markings as in Figure 39. The sections were immunostained for FoxP2 in green (FITC), and calbindin in white (Cy5). V1-INs are visualized naked in red (tdTomato). At E12.5 there are no V1-INs (A) leaving the progenitor area. FoxP2 (B) and calbindin-IR is present in more mature laterally located V1-INs. But there is little co-localization and while calbindin-IR V1-INs are located ventro-laterally, FoxP2-IR V1-INs are located more medio-dorsally. Bottom row contains superimpositions of various immunoreactivities. Scale bar; 100 μm in A, applies to all images.
In summary, the results confirm the time windows of neurogenesis for all V1 INs and Renshaw cells in particular, estimated with BrdU pulse-labeling studies in Aim1. It also suggests that MafB expression in Renshaw cells appears relatively late and only after the cells are close to their final locations. In contrast, FoxP2 is found in late born V1-INs from the moment they exit the cell cycle and before differentiation and migration out of the ventricular zone. Interestingly, calbindin seems similarly very early upregulated in V1-IN Renshaw cells and other early born V1-INs and therefore precedes MafB expression and constitutes the first phenotypic marker of early born V1-IN differentiation including the Renshaw cells. The results allow us to conclude that both calbindin-IR Renshaw cells and FoxP2 expressing V1-INs (that includes IaINs) start to phenotypically diverge from the moment they exit the cell cycle and start migration.

DISCUSSION

The main results described in this aim are: 1) MafB and FoxP2 are expressed in two distinct non-overlapping populations of V1-INs; 2) V1-INs expressing MafB include all calbindin-IR Renshaw cells; 3) Calbindin expression defines Renshaw cell precursors as they exit the progenitor zone while MafB expression is upregulated after a certain delay and only after Renshaw cells have migrated to their final locations; 4) FoxP2 is expressed in a population of late generated V1-INs that include a large proportion of the V1-derived IaINs that we can identify with the present histological criteria; 5) FoxP2 is expressed in late generated V1-INs as they exit the progenitor
zone. In conclusion, the V1-IN populations from which Renshaw cells and IaINs derive start differentiation from the moment they become postmitotic.

*Differences in reporter expression suggest early differences in the regulation of gene expression in different subgroups of V1 INs.*

Neither the *En1-Cre/Tau-lacZ* nor the *En1-Cre/Thy1-YFP* lines expressed the reporters early enough to allow analyses of the early differentiation of V1 INs. The first expression of the Tau-LacZ reporter was noted at E11.5 and for the Thy1-YFP reporter this date was E15.5. In both cases reporter expression at these ages was very weak, particularly in Renshaw cells. This lack of expression is most likely due to a developmentally regulated control of activity in the Tau and Thy1 promoters. Although it was not thoroughly quantified, the results also suggest that Renshaw cells are one of the last groups of V1-INs to upregulate these promoters. In particular YFP expression in the Thy1 line was not noted in Renshaw cells until relatively late (~E17) and only a subgroup of Renshaw cells expressed YFP at birth (P0). YFP expression spreads to all Renshaw cells by P5. The late upregulation of reporter expression in Renshaw cells suggest that genetic expression control mechanisms in Renshaw cells already differ from other V1-INs in the embryo. Thus, for analyses of E9.5 to E12.5 embryos we switched to the R26-tdTomato reporter line that exhibits a wider temporal window of reporter expression during development. We described only results from *En1-Cre/R26-tdTomato* litters, however the observations were replicated and largely confirmed in *En1-Cre/Tau-LacZ* litters from E11.5 age to E12.5 (not shown).
The first evidence of reporter expression in the spinal cord using En1-Cre/R26-tdTomato mice was E10.5. We confirmed that the genetic reporter expression system in these animals is indeed active as early as E9.5. Engrailed-1 expression is upregulated early in progenitor cells at the diencephalic/rhombencephalic boundary, a region that gives rise to the cerebellum. Progenitor cells in this area expressed abundant fluorescent protein in E9.5 embryos. In the E10.5 spinal cord the reporter was found in cells located at the border of the ventricular zone and in differentiating cells located more laterally in the mantle. The positions of weakly labeled cells at the lateral edges of the ventricular zone, exiting the progenitor zone, suggests upregulation of engrailed-1 in V1 INs as soon as they become postmitotic. If we consider an approximately 10 hour delay between S-phase BrdU labeling and the appearance of postmitotic neurons expressing specific transcription factors (Peng et al., 2007), our results match well the findings in Aim 1. BrdU was found to incorporate in V1-INs by E9.5. Following BrdU incorporation the cell needs to enter M-phase, divide, become postmitotic and start differentiation by expressing engrailed-1. Following steps include cre-recombination and reporter expression. Therefore a 24 hour delay between BrdU labeling and the appearance of the first reporter-labeled V1 INs is within the expected range. The results confirm that engrailed-1 is upregulated in early postmitotic differentiating V1-INs, but not in p1 progenitors (Matise and Joyner, 1997; Sauressig et al., 1999).

Transcription factor immunodetection in spinal cord neuronal subpopulations.

When analyzing the pattern of expression of transcription factors using antibodies, we need to be aware of possible confounding effects caused by antibody
specificity, temporal regulation of expression and possible epitope masking due to chromatin reorganization. MafB and FoxP2 immunoreactivities were always confined to the cell nuclei. This cellular localization is expected for transcription factors and suggests specificity. An optimal specificity test would be to perform these immunoreactions in mouse spinal cords lacking the proteins (i.e., expression knockouts). MafB and FoxP2 knockouts have been generated (Blanchi et al., 2003; Shu et al., 2005), but these animals are yet unavailable to us. Confirmation of the pattern of expression of both transcription factors was therefore done comparing immunolabeled cells in our sections with in situ hybridization genetic expression maps of the mouse spinal cord (Allen Brain and Spinal Cord Atlas; http://www.brain-map.org/). This expression maps contain data for the P4 and P56 mouse spinal cords.

The distribution of MafB mRNA expressing INs in this atlas is identical to the pattern of MafB protein immunolocalization we described at P0. Interestingly, in situ detection of MafB transcripts in the Atlas is described for both P4 and P56, while we were unable to immunolocalize the protein at P5 or later. Fast downregulation of MafB-expression in the postnatal spinal cord agrees with data provided by Geiman and colleagues in abstract form (Geiman et al., 2006). Mismatches between positive mRNA detection and negative immunolocalization have been described before and do not imply lack of antibody specificity. By contrast, the opposite situation in which positive immunolocalization is not matched by mRNA expression would have raised concerns about unspecific immunostaining, but this did not occur. Three explanations can be proposed for the observed mismatch with MafB mRNA expression in older spinal cords. First, high turn-over of MafB protein may result in a low steady-state
concentration, below immunodetection threshold. Second, lack of translation of the transcript into protein. Third, epitope masking obstructing access of the antibodies to the protein. To resolve these issues, we will need to test a variety of DNA denaturing and chromatin-unfolding methods before immunolocalization and corroborate the presence and levels of protein in spinal cords of different ages using Western blots. Alternatively, MafB expression could be followed by using a recently generated transgenic mouse in which a mafB 5'-upstream fragment directs expression of GFP mimicking MafB expression in cells, including ventral spinal cord neurons (Hamada et al., 2003). At present these experiments are beyond the objectives in this aim. It was fortunate that enough antigenicity was preserved at P0 and that at this age the immunolabelings correspond well with the distribution of cells expressing MafB mRNA in the P4 spinal cord, giving confidence that the immunolocalizations described herein accurately represent MafB-expressing INs in the spinal cord. However, we cannot be completely sure that the observed developmental downregulation of MafB immunoreactivity truly represents a downregulation of expression of this transcription factor in more mature INs, including Renshaw cells. We can be more certain that motoneurons express MafB early in development and then downregulate its expression. MafB in situ hybridization signals are positive in the developing motor pools of the chick embryo at day 4 (chick E4 corresponds roughly to E10 in mice as far as it relates to motoneuron development) (Eichmann et al., 1997; Lecoin et al., 2004) and were also detected from E10 to E13 in mouse spinal motoneurons (Eichmann et al., 1997; Hamada et al., 2003). MafB mRNA is however undetectable in postnatal motoneurons.
(Allen Brain and Spinal Cord Atlas), in agreement with the lack of immunolocalization signals in the postnatal spinal cord.

In conclusion, we identified four different cellular types that express MafB in the embryonic spinal cord: 1) Early developing motoneurons that then quickly downregulate MafB expression; 2) Ventral INs in which a majority are Renshaw cells and remain clustered in the Renshaw cell region through development; 3) A few dorsal V1-INs that do not express calbindin; 4) dorsal horn INs that arises from a dorsal progenitor area and spread all over the postnatal spinal cord dorsal horn. These groups match the MafB cells found at P0, except for motoneurons that seem to downregulate MafB before P0.

The distribution of FoxP2 immunoreactive neurons was compared to previous studies in the mouse embryonic spinal cord using both in situ hybridization (Shu et al., 2001, Dasen et al., 2008) and immunolocalization (Dasen et al., 2008; Morikawa et al., 2009). We found a similar onset of expression (at E11.5) and distribution of cells in E12 and E12.5 mouse embryonic spinal cords. Morikawa et al (2009) indicated that all ventral FoxP2 positive interneurons in E11 to E12.5 spinal cords also expressed En1-immunoreactivity suggesting they belong to the V1 population. Moreover, the proportion of V1-INs expressing FoxP2 increased from 18% at E11 to 59% at E12.5. One possible explanation for this increase is that they correspond with a population being added relatively late, as suggested in our studies. There are no previous studies on the distribution of FoxP2 positive neurons in the postnatal spinal cord. We found that at P5 only 33% of V1-derived INs express FoxP2, perhaps suggesting downregulation of FoxP2 in some V1-derived INs. If this was the case, the partial
labeling of V1-derived IaINs (66% of V1-derived IaINs express FoxP2) could be explained by downregulation before P5. Similar significant reductions in the number of FoxP2 positive spinal INs was observed from E13.5 to P0 by Morikawa et al. (2009), but the very significant growth of both spinal cord and neurons combined with the lack of stereological methods make interpretation of these data difficult. Alternative explanations are also possible. For example, the time course of En1 expression in individual V1-INs is unknown, but this information is important to interpret the data in temporal co-localization studies like the one described by Morikawa and colleagues. If En1 downregulates shortly after the cell is born, this could explain the selective enrichment of FoxP2 in En1 positive neurons at late developmental stages because earlier V1-INs would have downregulated En1 expression at this stage. Another difference between our estimates in postnatal spinal cords and those of Morikawa et al. in embryos is that we found many FoxP2-immunoreactive INs in the ventral spinal cord that do not belong to the V1 population. Given that ventral INs are largely generated before E12.5 the appearance of ventral FoxP2 interneurons that are not V1 can only be explained by late upregulation of this transcription factor in ventral non-V1 INs or by migration of dorsal FoxP2 populations into the ventral horn. Indeed, Morikawa et al. suggest that a population of dorsal dl2 INs express FoxP2 in early embryos and ends been localized in ventromedial positions. The relatively poor cellular resolution obtained with tdTomato fluorescent protein in tightly clustered early differentiating cells, did not allow us to perform accurate co-localization quantitative analyses. In the future thinner sections or immunocytochemical amplification of the reporter will need to be used to generate preparations from where accurate estimates can be obtained.
In summary, FoxP2 is expressed in a subpopulation of late generated V1-INs that includes many V1-derived IaINs identified histologically in the postnatal cord. Whether our results can be taken to suggest that FoxP2 is expressed by only a subpopulation of V1-derived IaINs is more difficult to conclude given the possibility that by P5 many V1-derived INs could have downregulated this transcription factor. Alternatively, these analyses should be done earlier, but at present it is not possible to identify IaINs at early developmental stages because the criteria we use for identification is based on synaptic connectivity and synaptogenesis in the spinal cord starts at embryonic ages later than the ones we studied here. On the other hand, if our data at P5 truly indicates heterogeneity in FoxP2 expression in the IaIN population this should not be too surprising given the diversity of IaINs in their origins (Siembab et al., 2010), morphological characteristics (Rastad et al., 1990), trajectories of their axons and electrophysiological properties and inputs (reviewed in Jankowska, 1992). Therefore it is possible that our data at P5 indicates that FoxP2 expression is temporally regulated in a different manner within different groups of V1-derived IaINs or not expressed at all in some. Despite these interpretation caveats, FoxP2 does allow us to investigate the early differentiation of a group of V1-INs that is distinct from Renshaw cells/MafB-expressing V1 INs and includes, at minimum, a sizable proportion of V1-derived IaINs.

Specifity of transcription factor expression in V1’s, Renshaw cells and IaINs

The results strongly suggest that neither FoxP2 nor MafB can be regarded as transcription factors exclusively expressed by V1-INs, IaINs or Renshaw cells. Thus,
we cannot conclude that these transcription factors alone control the differentiation of each of these INs. It is more likely that a combination of these with other unknown transcription factors expressed either simultaneously or sequentially might be responsible for their specific differentiation as occurs during specification of motoneuron subtypes (Jessell, 2000; Price and Briscoe, 2004). A “transcription code” for the specification of adult IN subtypes is at present unknown for any spinal cord population. What could then be the role of MafB and FoxP2?

Knockout MafB and FoxP2 animals display severe motor abnormalities, but this cannot be ascribed solely to the loss of subgroups of V1 spinal INs since these transcription factors are also important for the developmental of many other brain regions. MafB is involved in the differentiation of monocytes and macrophages, specification of rhombomeres 5 and 6 in the brainstem and the development of rhythmogenic cells in the respiratory cells of the preBötzinger complex of the medulla. An old X-ray induced mutation named Kreisler (Hertwig, 1942) is now known to inactive MafB expression in some cells including those in the medulla, blood cells lines and a number of other peripheral cells (but not in respiratory neurons). These animals survive but have deformed vestibular apparatus and cochlea and are deaf, cannot swim, run in circles with head tossing and also have a compromised immune system (reviewed in Eichmann et al., 1997). In contrast, complete knockout of MafB results in animals that die due to respiratory apnea just after birth because the deletion of critical INs of the respiratory center in the preBötzinger complex (Blanchi et al., 2003). To our knowledge there are no published analyses of spinal cord development in these animals. One personal communication at a meeting (Symposium on Neurons and Networks in
the Spinal Cord. University of Wisconsin – Madison. June 2009) from the lab of Dr. Martyn Goulding (Salk Institute. San Diego, CA) suggested that Renshaw cells start normal differentiation in early embryos but undergo apoptosis in late embryos in MafB knockout animals. If these results are confirmed, MafB could be a critical factor for the maintenance of Renshaw cells but not for their early specification and this would agree with the timing of its expression, as described in here.

FoxP2 is also involved in many different developmental processes, and has now gained fame due to its genetic linkage with human language deficits and with vocal learning in other species (reviewed in Vargha-Khadem et al., 2005; Fisher and Scharff, 2009). However, FoxP2 is also widely expressed in many tissues and cells during development and in the adult suggesting many different functional roles (Shu et al., 2001). FoxP2 knockout mice express many severe motor deficits and an underdeveloped cerebellum (Shu et al., 2005). These animals eventually die by P21, although the cause of death was not fully investigated. Given the widespread functions of FoxP2 it would be desirable to have better control for gene deletion in specific cells and at specific times. This is now possible because the development of transgenic mice carrying a conditional FoxP2 allele that can be removed upon Cre-mediated recombination (French et al., 2007). To our knowledge there are no studies on the role of FoxP2 in interneuronal development, however it is interesting that the related transcription factor FoxP1 is highly express in motoneurons during development where it controls pool specification and motoneuron spinal cord position and connectivity (Dasen et al., 2008). If FoxP2 had a similar function in defining pools within IaINs, or would explain the uneven distribution of FoxP2 in this population at P5.
Differentiation of early and late V1 populations starts as soon as they are born.

The principal question we asked in this aim was whether or not early and late generated V1-INs that include, respectively, Renshaw cells and IaINs start their differentiation as soon as they are generated. The alternative possibility was that they remain relatively undifferentiated until a later time, for example until the onset of synaptogenesis. This second possibility could imply that integration into synaptic circuits is a major factor in determining fate and differentiation. Our results falsify this possibility and suggest that different groups of V1-INs commit to differentiation pathways as soon as they start to leave the progenitor area and before synaptogenesis. Interestingly, FoxP2 is expressed in late V1-INs very early, as they exit the progenitor zone, while MafB is upregulated relatively late in earlier generated Renshaw cells, and only after these cells have reached their final positions. This does not mean, however, that Renshaw cells start differentiation relatively late. Early generated V1-INs express calbindin as they exit the progenitor zone and calbindin expression seems unique to this group of V1-INs not being replicated by late V1-INs expressing FoxP2. Therefore, the first phenotypic marker we identified for early V1-INs is calbindin, while FoxP2 appears to be widely expressed in late generated V1-INs.

Within the early and late V1 populations it is likely that there are also subdivisions. For example, calbindin-IR V1-INs also included the big upper lumbar cells that according to aim 1 are generated overlapping with Renshaw cells. The early calbindin expressing population is also likely to include other V1-IN groups that downregulate calbindin during late embryo and early postnatal development (Siembab
et al., 2010). Perhaps many of the calbindin V1-INs located medially to the motoneuron pools belong to this group. It is interesting that MafB expression seems relatively restricted to Renshaw cells within the calbindin-IR V1 group and that MafB is upregulated only after Renshaw cells reach their final locations ventral and lateral to the motor pools (during the morphogenetic movements related to the growth of the spinal cord in late embryo this location will end being ventromedial to the motor pools in the lumbar segments). In parallel with the known hierarchical organization of transcriptional codes in motoneurons, MafB could represent a relatively late inductor of properties that differentiate Renshaw cells from other groups of early generated V1-INs that express calbindin in embryo. Interestingly this temporal regulation of MafB expression appears to be specific to V1-INs because dorsal horn cells that also express MafB upregulate its expression as they exit the progenitor zone.

Overall, the evidence in this aim suggests that differentiation of IN subtypes from the canonical V1 group occurs very early, perhaps determined by genetic-inductive processes before circuit formation. The mechanisms that trigger early developmental programs that specify subgroups of V1-INs are unknown but it was observed that MafB expression was not expressed in developing Renshaw cells until they reached their final position. Therefore we speculate whether their migratory behavior could provide insights into mechanisms of Renshaw cell differentiation. In aim3 we investigated whether or not Renshaw cells follow a distinct migration pathway that positions them among motor axons.
AIM 3

CHARACTERIZATION OF THE MIGRATORY PATHWAY OF RENSHAW CELLS AS DISTINCT TO THAT OF OTHER V1-INS
INTRODUCTION

The migration route taken by newborn neurons can affect their development by restricting their potential for differentiation or inducing certain characteristics as they move through tissues. This influence of migration has been more thoroughly studied for neural crest cells that are generated at the interface between the skin ectoderm and the neural tube and migrate throughout the body giving raise to all the peripheral nervous system, including the sensory ganglia and the autonomic nervous system. For example, sympathetic neurons leave the neural crest and migrate towards the aorta where they acquire a noradrenergic phenotype induced by aorta-released BMPs that upregulate the expression in the migrating neurons of transcription factors that control noradrenergic genes (Shah et al., 1996).

In the central nervous system, birth-date, migration, final position and phenotype are closely interrelated as shown in the laminar segregations of retinal and cerebral cortex neurons (in cortex McConnell and Kaznowski, 1991; McConnell, 1995; Butt et al., 2007; Leone et al., 2008; Wonders and Anderson, 2006; in retina Watanabe and Raff, 1990; Cepko et al., 1996; Voinescu et al., 2009). Although much is unknown about the exact molecular mechanism, experiments with progenitor cells implanted in tissues or co-cultured with cells of younger or older age suggest that they contain internal cell programs regulated by internal clocks that make them more or less susceptible to environmental influences that also change with time as the nervous system develops. As a result the developmental potential of progenitor cells gets restricted with time and they generate different cell types that become located at different positions (see review: Leone et al., 2008; see in retina: Watanabe and Raff,
Many of the temporal changes that occur in progenitors are related to sequential changes in transcription factor expression and some of these are known to directly influence migration (Butt et al., 2007; Leone et al., 2008). In conclusion, the interaction between migration and differentiation seem to be bidirectional, some cells are biased towards certain migration routes and at the same time the route of migration and local environment further specifies novel cellular properties.

Spinal cord LMC motoneurons exhibit similar relationships between birth-date, migration, transcription factor expression and phenotype. As reviewed before, a retinoid signal provided by one subset of early-born spinal motoneurons specifies subcolumnar identity in later-born motoneurons as they migrate past each other (Sockanathan and Jessell, 1998). Furthermore, visceral motoneurons of the sympathetic preganglionic motor column settle in the intermediate horn at thoracic levels and this position is determined by expression of the transcription factor Isl2, which is key for their migration and future differentiation (Thaler et al., 2004). It is possible that similar mechanisms are at work for differentiation of spinal ventral INs, but this is yet unknown. In an early study a number of different directions of migration were proposed for motoneurons and ventral INs, with most migrating in a lateral and ventral direction (Leber and Sanes, 1995). It is now clear that cells from different canonical subtypes (V0, V1, V2, V3) display preferential migration directions in the spinal cord and end located in different positions (see review in background). However, within these canonical groups, further subdivisions according to migratory pattern have only been suggested for the V3 group and their significance is unknown (Zhang et al., 2008). Within adult V1-derived INs, Renshaw cells display a unique position, known in
the past as the “Renshaw cell area” located in ventral LVII and LIX (Thomas and Wilson, 1965; Willis 1971; Alvarez and Fyffe, 2007). In Aim 2 we found that this location is correlated with positioning at the ventro-lateral edge of the developing embryonic spinal cord. We therefore asked in here whether this is related to a specific migratory pathway for early-born Renshaw cell precursors that in addition could explain their close relationship with motor axons and may be also important for further development of Renshaw cell properties. For example, MafB expression seems upregulated in Renshaw cells only after they reach their final position (Aim2).

Previous studies suggested that V1-derived INs leave the progenitor area as soon as the last cell cycle division is completed (Saueressig et al., 1999) and therefore early and late born V1-INs should start migration at different times. This implies possible differences in routes and interactions within a developing neural tube that changes with age. Based on Aim1 results we know that Renshaw cells leave the cell cycle around E9.5 and E10.5, coinciding with the time in which motoneurons become postmitotic in the mouse spinal cord (Sims and Vaughn, 1979; Holley et al., 1982; Wentworth, 1984). The early expression of calbindin in Renshaw cell precursors (Aim2), just as they migrate away from the ventricular zone, could suggest that they are already at least partially differentiated towards some aspects of the Renshaw cell phenotype and this could include a specific migration route. It has also been suggested in the early spinal cords of the mouse (E9.5 to E11.0) and the chick (S18 to S23) that cytoplasmic extensions from both motoneurons and radial glia connect the ventricular zone with the external surface of the neural tube (Holley, 1982). Developing Renshaw cells could use these pathways for lateral migratory guidance. Therefore, we suggest
that because motoneurons and Renshaw cells have similar birth dates, their migratory timing and pathways might be related and in part responsible for some aspects of their future differentiation. Indeed in an early study looking at V1 cells at different embryonic stages it was suggested that V1 cells first move laterally within the neural tube and then start to migrate ventrally positioning themselves medial to motor pools (Matisse and Joyner, 1997). The results in aim 2 clearly suggest that developing Renshaw cells are different in that their final position in the embryonic spinal cord is ventro-lateral to motor pools, not medial. No previous study has directly analyzed these migratory patterns or established differences among the different groups of V1-INs. Moreover, there are a number of different pathways available for putative Renshaw cells to settle into their final positions between motoneurons and the ventral root. These include pathways around motoneurons dorsally or ventrally or even through the motoneuron pools. Each migratory route could suggest different possible interactions with the maturing motor pools.

We therefore hypothesize that a subset of early-generated V1-INs which will become the Renshaw cell pool is attracted toward the motor axon exit and they could interact with motoneurons during the migration process in a number of different manners. In contrast, later born V1-INs stop their migration medial to motoneurons and these could become other types of premotor inhibitory INs. We will test this hypothesis by analyzing the relationships of migrating Renshaw cell precursors with motoneurons. For these studies we used En1-Cre/R26-tdTomato embryos and combined V1 genetic labeling with calbindin-immunoreactivity to distinguish Renshaw cell precursors within the V1 group. The sections were also immunostained for either a motoneuron marker.
(the transcription factor, islet 1) or a marker of neurites in immature neurons (Tuj1 monoclonal antibodies against class III beta-tubulin, give ref) to label the developing ventral roots.

**MATERIAL AND METHODS**

*Animals and Tissue extraction*

Time pregnant females were obtained as before to obtain embryos from E10.5 to E12.5. We analyzed E10.5 (n = 4 embryos from 2 litters), E11.5 (n = 7 embryos from 2 litters), E12 (n = 4 embryos from 2 litters) and E12.5 (n = 2 embryos from 1 litter), embryos generated by crossing En1\(^{Cre/+}\) heterozygotes with the CAG-Rosa26-lox-STOP-loxp-tdTomato-WPRE reporter line. Timed pregnant females were perfused as described in Aim 2 and the embryos removed and fixed *in toto* overnight and then cryoprotected in 30% sucrose. Positive embryos (expressing tdTomato in En1+ cells) could be easily identified by a large area of red fluorescence observed in the midbrain region (see Aim 2). Embryos were cut in a cryostat at 20-30\(\mu\)m thickness and collected in subbed slides. Slides were stored at -20\(^\circ\)C until processed.

All animal procedures were carried out according to NIH guidelines and were approved by WSU LACUC.

*Immunolabeling.*

For these experiments rabbit polyclonal antibodies (1:500, Swant, Bellizona, Switzerland), were combined with monoclonal mouse antibodies against Islet1 or Tuj1 Immunoreactive sites were revealed with fluorochrome-conjugated (FITC, Cy3, and
Cy5) donkey species-specific secondary antibodies (1:50, Jackson Laboratories) or Alexa-405 (Invitrogen) as previously described and all immunofluorescent preparations were coverslipped with Vectashield (Vector Labs).

Table 4. Antibodies used in Aim 3 and labeling specificity

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Host species</th>
<th>Dilution</th>
<th>Company</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Swant,</td>
<td>No labeling in KO tissue</td>
</tr>
<tr>
<td>D28K</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Islet1</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:100/1:50</td>
<td>Hybridoma Bank, Iowa</td>
<td>Only known embryonic spinal islet1 cells labeled</td>
</tr>
<tr>
<td>Tuj1</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:500</td>
<td>Chemicon</td>
<td>Only present at early embryonic stages</td>
</tr>
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Analysis: Images were obtained in an Olympus FV1000 confocal microscope at 10X1, 20x1 and 60x1. Image composition and presentation were done as in aims 1 and 2.

RESULTS

Analysis of embryonic spinal cord sections from E10.5 to E12.5 helped us generate a plausible scheme of V1 migratory pathways based on static images. At E10.5 few tdTomato-labeled V1-INs are present in the embryonic spinal cord. Most are calbindin-IR and located in close proximity to the spinal cord surface and just dorsal to Islet1-IR motor pools (Fig. 47). At this age there are very few differentiating cells in the mantle layer of the embryonic spinal cord and therefore these cells, although located in apposition to the lateral surface they are not far from the ventricular zone where they
**Figure 47. Locations of calbindin-IR V1-INs with respect to Islet1-IR motoneurons in the embryonic spinal cord at E10.5.** Low (A to F) and high (G to L) magnification confocal images of spinal cords from the En1-Cre/R26-tdTomato mouse line. The solid line delineates the boundaries of the embryonic spinal cord, while the dotted line marks the midline and progenitor area. Sections were immunolabeled for Islet1 in green (FITC) and calbindin in white (Cy5). V1-INs were visualized naked (without any antibody enhancement) in red. At E10.5 the V1-IN population (A and G) is located right above the motoneuron pool (F and L). Motoneurons (Islet1 positive) are already located ventrally at this age (B and H). In these images we also confirmed that at E10.5, all V1-INs present are calbindin positive (D and J). Some calbindin-IR cells have bipolar morphologies and some are oriented medio-laterally. However the most striking groups are located most laterally, have unipolar morphologies send out projections ventrally to a very specific area in the ventral horn (white arrow in I). Projections follow the most external path or infiltrate the motor pools. Many end in growth cones which are frequently clustered at this ventro-lateral location (J and K). Scale bar; 50μm in A and G (B to F are at the same magnification as A; H to L are at the same magnification as G).
originated and they are at a similar dorso-ventral level. The simplest interpretation of these images is that this early group of INs did not move much laterally but just exited the proliferative zone and started the formation of the mantle layer just above the motor pools, which are settling more ventrally. This might suggest that this early group of V1 INs is possibly one of the first IN cell groups that start differentiation in the early spinal cord. At this age most are unipolar or bipolar neurons with short processes. The cells located more medially are frequently bipolar and located at the transition zone between the progenitor area and the mantle layer. The border between these two regions is better visualized in sections immunostained against Tuj1 (Fig. 48). In these sections some calbindin-IR V1-INs are clearly identified outside of the proliferative zone (i.e., Fig. 48 C, I, and F). In bipolar cells one process is oriented laterally, toward the surface of the neural tube, and the other is shorter and directed medially, likely retracting from the ventricular area. More laterally located cells are presumably more mature neurons generated earlier. These cells are mostly unipolar with a clear process directed ventrally (Figs. 47G-L, 48D-I). These ventral processes course in between the outer edge of the spinal cord and the Islet1-immunoreactive motor pools or through the motor pools. In Tuj1-labeled sections these processes seem to terminate in front of the motor root exit area (Fig. 48D-I) and frequently end in bulbs that resemble growth cones (arrow in Fig. 47I).

At E11.5 there is a significant increase in the number of V1 INs (Figs. 49 and 50), but still the majority are calbindin-IR (as described in Aim 2). They are distributed in two groups. The more striking population of calbindin-IR V1 INs are tightly clustered in a thin layer surrounding the most lateral and ventral corner of the spinal
Figure 48. Calbindin-IR V1-INs project ventrally to the area right next to the motor axon exit zone in the E10.5 spinal cord. Low (A to C) and high (D to I) magnification confocal images of an embryonic spinal cord from a wild-type animal. Markings as in Figure 47. Sections were immunolabeled for Tuj1 (Neuron-specific class III beta-tubulin, red, Cy3) and calbindin (green, FITC). A) Tuj1-IR targets microtubules of undifferentiated neurons and labels mostly processes including the axons. Dorsal roots and ventral roost are well defined in these preparations (see Figure 4). The progenitors are unlabeled by Tuj1 antibodies. B) Calbindin-IR cells (E and H) send projections to the area in front of the motor axon exit as visualized with Tuj1 immunolabeling (C). D to I), High magnification images showing the relationship between the ventral root exit zone and the ventrally directed processes sent by calbindin-IR neurons in the E10.5 spinal cord. Scale bar; 50 μm in A and D (B and C are at the same magnification as A; E to I are at the same magnification as D).
Figure 49. Location of calbindin-IR V1-INs with respect to Islet1-IR motoneurons in the embryonic spinal cord at E11.5. Low (A to F) and high (G to L) magnification confocal images from an En1-Cre/R26-tdTomato mouse. Markings as in Figure 47. Sections were immunostained for Islet1 (B and H, green, FITC) and calbindin (C and I, white, Cy5). V1-INs (A and G) are shown in red due to the presence of a red fluorescent protein in their cell bodies. By E11.5 calbindin-IR V1-INs are located in two distinct areas. A more lateral group is at the very edge of the spinal cord and seems to have followed the projections seen at E10.5. A second group is located medial or intermingled with motoneurons. Scale bars; 50 μm in A and G (B to F at the same magnification as A; E to I at the same magnification as D).
cord. These cells appear to correspond to presumed developing V1 Renshaw cells that were located more dorsally at E10.5. In Tuj1-labeled sections these lateral cells are found in close relationship with the ventral root exit (Fig. 50). The medial group of V1 INs is located far from the ventral roots and positioned medial or intermingled with islet1-immunoreactive motoneurons. A large proportion of these V1 cells are also calbindin immunoreactive. At this age most calbindin-IR V1 cells in both groups are multipolar with several neurites emerging from their cell bodies. The cell bodies of the lateral group are spindle-shaped while those of the medial group are more rounded. At this age there are few calbindin-IR V1-INs close to the progenitor area, these few cells are now oriented radially in a ventro-lateral direction pointing towards the motor pools. Medial V1 cells are not as tightly clustered as the lateral group because the basal plate (or developing ventral horn) medial to motoneurons has added by now many other cell types in addition to V1-INs.

In addition, at this age we observed V1 calbindin-IR putative axons oriented longitudinally and therefore cross-sectioned in the incipient marginal layer (future white matter). These axons are located just external to the lateral group of calbindin-IR V1 INs (Figs. 49G-L, 50D-I). This suggests that early calbindin-IR V1 axons (many of which probably arise from developing Renshaw cells) contribute also to pioneer the ventral funiculus. The majority are positioned just medial to the ventral root exit. In Tuj1-immunostained sections it is clear that a large contribution to the early white matter comes from commissural axons that cross just ventral to the floor plate in a region that will later become the ventral white commissure. As already suggested by the early neuroanatomists (Cajal, 1995), the formation of the white matter starts
Figure 50. Calbindin-IR V1-INs, presumably Renshaw cells, position their cell bodies in front of the motor axon exit by E11.5. Low (A to C) and high (D to I) magnification images of ventral portion of the spinal cord in a wild-type animal. Markings as in Figure 47. Sections were immunostained for Tuj1 (red, Cy3) and calbindin (green, FITC). At E11.5 a group calbindin-IR cells (B, E, and H) are located in front of the motor axon exit (A, D, and G) where they sent their projections at E10.5. The remaining calbindin-IR cells are located in between the motor pools or more medially (C, F, and I). Scale bars; 100 μm in A (B and C have same magnification); 50 μm in D (E, F, G, H, and I have same magnification).
ventrally and at this age there is yet no evidence of axons accumulating in the dorsal marginal layer.

These observations suggest that by E11.5 most developing V1 Renshaw cells have established a close spatial relationship with motor axons. The nature of calbindin-IR V1 cells found interspersed in between the motor pools or just medial to them is more difficult to interpret. They could represent late Renshaw cells traveling in between motoneurons towards the motor axons or alternatively could represent populations of V1 INs that settle away from the Renshaw and motor axon region and become a different class of V1-INs. They could become V1-INs that later downregulate calbindin immunoreactivity (Siembab et al., 2010) or from part of the novel groups of calbindin-IR V1 cells described in Aim 1 and located more dorsally. Whether they are migrating or not can only be fully proven in future time-lapse studies, however, a multipolar appearance and extension of dendrites occurs in motoneurons and INs only after they appear to reach their final destinations and stop migration (Wentworth, 1984a,b). Most of these cells show well developed dendrites and morphology and therefore the more plausible interpretation is that they are not migrating Renshaw cells.

At E12.0 it is still noticeable how some V1-INs are still leaving the progenitor zone (Fig. 51), but these late generated cells are not calbindin-immunoreactive, as also explained in Aim 2. In addition, non-V1 calbindin-IR neurons start to appear more dorsally (arrows in Fig. 51). In the ventral horn the two same groups consisting of a lateral differentiating clustered group of calbindin-IR V1 Renshaw cells and a more medial calbindin-IR V1 group are still visible. By E12.5 no more V1 cells are leaving the progenitor cell area (corroborating our conclusions from BrdU analyses in Aim1).
Figure 51. Location of calbindin-IR V1-INs with respect to Islet1-IR motoneurons in the embryonic spinal cord at E12.0. Low (A to F) and high (G to L) magnification confocal images of spinal cord from the En1-Cre/R26-tdTomato line. Markings as in Figure 47. Sections were immunostained for Islet1 (B and H, green, FITC) and calbindin (C and I, white, Cy5). V1-INs (A and G) are shown in red. At E12.0 the V1-INs are grouped in pools (A and G). Calbindin-IR V1-INs are located with respect to islet1 motoneurons in similar relative positions as in E11.5. In addition a new group of calbindin negative V1-INs is added at this age and this group is located medio-dorsal to islet1-IR motoneurons. Scale bars; 100 µm in A (B to F are at same magnification); 50 µm in G (H to L are at the same magnification).
and calbindin-IR V1 INs are fixed at the same positions in relation to islet1-labeled motoneurons and Tuj1-labeled ventral roots (Figs. 52 and 53). At E12.0 and E12.5 V1-INs display a greater variety of morphologies, including dendritic arbors with medio-lateral and dorso-ventral orientation. At this age the cellular density has also increased considerably and the commissural axons (best visualized in Tuj1-immunostained sections) divide the mantle layer into a lateral and medial region. Most V1-INs with multipolar morphologies are located in the lateral regions in close proximity with the motor pools. At E12.5 the pool of Renshaw cells is located in a more ventral and less lateral positions. This change of location seems related to morphogenetic movements of the spinal cord due to its increase in size and cellular density. Thus, calbindin-IR V1 Renshaw cells and ventral roots move together and there is no change in their spatial relations. At the same time the marginal zone significantly increases in thickness by the addition of further axons many of which express tdTomato but lack calbindin, suggesting that V1 axons from late born V1-INs have now reached the marginal layer. Renshaw cell axons (tdTomato and calbindin) remain closer to the border between the mantle layer (future grey matter) and the marginal layer (future white matter). At E12.5 there is considerable growth of the dorsal marginal layer that becomes invaded with many axons (Fig. 53A). V1 axons travelling in the white matter do not extend collaterals back to the developing motor columns and therefore there is no yet evidence of terminal arborizations and synaptic varicosities.

The above descriptions correspond to spinal cord regions located at the level of the lower limb buds and therefore correspond to mid to high lumbar locations. Interestingly, cross sections through the more caudal neural tube demonstrates a
Figure 52. Location of calbindin-IR V1-INs with respect to Islet1-IR motoneurons in the embryonic spinal cord at E12.5. Low (A to F) and high (G to L) magnification confocal images of the ventral horns of E12.5 spinal cords. Markings as in Figure 48. Sections were immunolabeled for Islet1 (B and H, green, FITC) and calbindin (C and I, white, Cy5). V1-INs (A and G) are shown in red. At E12.5, the relationships between V1-INs (A and G) and Islet1 motoneurons is similar than at E12.0. Scale bars; 100 μm in A (B to F are at same magnification); 50 μm in G (H to L are at the same magnification).
Figure 53. Calbindin-IR V1-INs retain their position close to the motor axon exit at E12.5. Low (A to E) and high (F to L) magnification confocal images of an embryonic spinal cord from wild type animal. Markings as in Figure 47. Section were immunostained for Tuj1 (red, Cy3) and calbindin (green, FITC). At E12.5, a group of calbindin-IR cells (B, D, H, and K) remains located in front of the motor axon exit (C, E, I, and L) with some other cells located further away in between the motor pools or medial to the motor pools. Scale bars; 100 μm in A (B, C, D, E, and F are at same magnification); 50 μm in G (H, I, J, K and L are at same magnification).
significant delay in the generation of V1-INs. In caudal cross sections early generated calbindin-IR V1 INs are still exiting the progenitor layer and none have yet reached their final locations in front of the ventral root exit (Fig. 54). Thus, despite the lack of differences in V1-IN generation between upper and lower lumbar segments (Aim 1) significant rostro-caudal temporal gradients in the emergence of V1-INs exist nevertheless. Neurogenesis and migration of more caudal V1-INs seems considerably delayed compared to lumbar levels.

In summary, the early born Renshaw cells appear to follow a unique migratory pathway. First, at the time of birth they immediately reach the lateral surface being among the first to exit the ventricular zone. Second, these cells extend process that travel ventrally towards the ventral root exit. Third, the cells appear to follow these processes and settle at the ventral root exit. By E11.5 Renshaw cells have reach their final positions between exiting motor axons. In contrast, other V1-INs are continuously added from E10.5 to E12.0, but these cells do not migrate in between motor pools to reach the external surface of the embryonic spinal cord. Instead, they take a ventro-lateral migratory direction towards the motor pools and settle just medial of the motoneuron pools or in between them.

DISCUSSION

Our analyses of V1-derived IN migratory routes are based on static confocal images obtained at different embryonic ages. These images provided enough information to propose 1) a possible migratory mechanism and 2) different routes of
Figure 54. V1-INs in caudal segments of the spinal cord at embryonic E12.5 resemble earlier embryonic stages of lumbar regions. Low (A to E) and high (F to L) magnification confocal images of an embryonic spinal cord from a wild type animal. Sections were immunostained for Tuj1 (red, Cy3) and calbindin (green, FITC). Caudal embryonic section from E12.5 embryo resembles an E10.5 (A to C) of more rostral segments. At this age and level we can observe calbindin-IR cells exiting the progenitor area, sending their projections to the motor axon exit and with cell bodies still located dorsal to the motor axon exit region (B, C, F, I, and L). Scale bars: 100 μm in A and D (B, C same magnification as A; E and F same magnification as D). 50 μm in G (H, I, J, K and L have same magnification).
migration for Renshaw cells and other V1-derived INs. This different route of migration suggests a special relationship between developing Renshaw cells and motor axons.

However, further analyses using time-lapse microscopy will be needed to confirm these migratory pathways. The use of multiphoton confocal microscopy for this time lapse studies would be preferred over single photon confocal microscopy because it will better preserve fluorochromes and cells for the long exposure times that we expect will be necessary.

*Interpretation of static images into a model of migratory behavior.*

We believe that our static images suggest a clear migratory path for Renshaw cells. In other parts of the nervous system, cell migration *in situ* has been demonstrated to proceed through neurite elongation followed by nuclear translocation. Pyramidal cells of the neocortex migrate along radial glia (Hatten, 1990) by extending a leading neurite towards the surface of the cortex while retracting a trailing neurite that initially was attached to the ventricle, while the leading edge moves forward and the trailing edge is retracted the nucleus is pushed in the forward direction (Noctor et al., 2001).

More dramatic are time lapse images of cortical INs *in vitro* in which migratory movements occur through initial extension of a neurite followed by nuclear translocation to its leading edge when this stops. Then the leading neurite is extended again in another cycle of neurite extension and nuclear translocation. This stop-and-go movement continues until the neurons stop migrating (Bortone and Polleux, 2009).
Our images suggest a similar mechanism for calbindin-IR V1 Renshaw cell precursors in the spinal cord. Initially the cells leaving the progenitor area have a medio-lateral bipolar morphology in which the lateral extension seems to be a leading edge in search for the surface of the neural tube while the medial neurite appears as a shorter trailing end. We don’t know if at this point the cells follow radial glial surfaces, but because these are among the first neurons to be generated, above the motor pools, their final position is very close to the point they exit from the progenitor area, and therefore radial glial interactions might not be necessary or essential. According to the model of neurite extension-nuclear translocation, once the lateral “leading” neurite reaches the surface, the nucleus should be translocated to this lateral position at which point the neuron becomes unipolar by retracting the trailing end. Then the leading neurite turns 90° in the ventral direction. Ventrally directed neurites that can be followed to their end-bulb within single sections always seem to stop in front or close to the ventral root exit. These end-bulbs resemble growth cones, however this will need to be further confirmed with more specific markers like the growth protein GAP-43 (Goslin et al., 1988). The route these neurites followed to get there can be deduced by the location of the neurite stem between the cell body and the end-bulbs. Sometimes this follows a lateral course between the spinal cord surface and the motor pools, others it goes through the motor pools. In any case it seems these neurites are attracted by the ventral root exit region. Following the model, the nucleus of the cell is translocated to the ventral root exit region once the tip of the neurite reaches this position. Indeed, by E11.5 many of the cell bodies of these cells have relocated to the ventral root exit region. The route they might have followed is then given by the course of the neurites
as seen in E10.5 spinal cords. It is unlikely that this ventrally directed migration follows any scaffold from motoneurons or radial glial cells. First, the pathway followed by these neurites is quite tortuous. Second, their direction is orthogonal to radial glia (see Cajal, 1995 for a representation of radial glial orientation in the spinal cord at this age).

In summary, our images suggest that Renshaw cell migration towards the ventral root is a two step process in which the cells first position themselves at the lateral edge of the spinal cord above motoneurons and then move to the ventral root exit in a second step. This model clearly suggests the presence of a powerful attractor for these cells in the ventral root exit zone.

**Comparison with the migration of non-Renshaw V1 interneurons**

The early migration of ventral INs (Leber and Sanes, 1995) and V1-INs (Matise and Joyner, 1997) was initially proposed based also on static images from different developmental stages. These authors postulated that the neurons leave the progenitor area and migrate medio-laterally to the surface and then most migrate ventrally, settling mostly medial to motor pools. Our analyses confirm this overall migratory behavior, however the migration of Renshaw cells is quite different from that of other V1-INs. The lack of markers for specific subgroups of V1-INs might have resulted in a global description of migratory routes that includes those taken by several different subpopulations. Non-Renshaw V1-INs seem to migrate in a ventro-lateral radial path. This makes sense since this is the main orientation taken by radial glial cells after the growth imposed by cell additions in the ventral spinal cord (Cajal, 1995). These cells then stop far away from the ventral root exit zone. There are several possible
explanations. One is that they have lower chemotropism for this region, another that they cannot pass the motor pools which then will be acting like a barrier. Finally, it is possible that a signal to stop migration is triggered before they can reach the ventral root exit zone. In this case signals that propel migration could be as important as guidance molecules for the final position of the cells.

**Mechanisms that permit migration**

Recently, Bortone and Polleux (2009) demonstrated that prior to synaptogenesis, migrating cortical INs respond to ambient GABA, likely released in a paracrine manner. During migration, GABA strongly depolarizes the neurons and stimulates motility, but later as the potassium-chloride cotransporter KCC2 is upregulated, GABA signaling is interpreted as a stop signal. This study pointed out the importance of distinguishing between molecules that attract INs to their final destination from permissive mechanism that allow the INs to actually migrate.

In the spinal cord, KCC2 is first detected in the developing ventral horn at E11.5 (Delpy et al., 2008) and therefore it is possible that in resemblance to the mechanisms found in the cortex GABA provides a signal to stop migration once a certain level of internal chloride concentration is reached. However, it is clear that at this early age the effects of GABA and glycine are still depolarizing in ventral neurons (Scain et al., 2010). It will be interesting to study the relationships between chloride homeostasis and IN migration in the spinal cord ventral horn. It is important to note that V1-INs are a likely source of paracrine GABA release in this early spinal cord. In this sense, they might autoregulate their own migration.
Mechanisms and significance of Renshaw cells ventral root chemotropism

It is unclear what signals attract developing Renshaw cells into the ventral root, but our observations indicate it is a powerful one. One possibility is that the signals are provided by the motor axon themselves, for example released acetylcholine. But there are also alternative possibilities since the presence of molecular cues in this region is necessary for the motor axons themselves to pierce the surface of the neural tube precisely at this point. Whether the signals are provided by some intrinsic motoneuron factor or by other cells outside the neural tube surrounding or in the vicinity of this area is unknown. What is clear is that this attractor positions developing Renshaw cells in the right place to interact with motor axons and this could be the basis of the specific synaptic coupling between motor axons and Renshaw cells. Up-to-date and despite some evidence of possible alternative INs that could be more weakly linked to motor axons (Machaceck and Hochman, 2006; O’Donovan et al., 2010), Renshaw cells remain the main spinal interneuronal target of motoneurons. The results from this aim suggest that their special birth-date and migratory route might be responsible for establishing this strong linkage. Moreover this might influence their final differentiation since they upregulate the transcription factor MafB once they reach this position.

A migration dependent on birthdate that then defines specific patterns of synaptic connections has already been suggested in other parts of the central nervous system. One example are gonadotropin-releasing hormone (GnRH) neurons from the hypothalamus (Jasoni et al., 2009). These authors postulate that first generated neurons settle more rostrally than later-generated neurons and this defines their activity and
modulation in the adult. Furthermore, recent studies in cortical INs in the mouse telencephalon have shown that the transcriptional control of interneuronal development results from the interplay between migration and time of differentiation (Butt et al., 2007; Leone et al., 2008).

Overall our experiments suggest there are two main groups of V1-derived INs, early and late generated from the p1 domain. The early generated V1-derived INs, which include among others Renshaw cells and big-calbindin-IR cells, are generated in between E9.5 and E10.5. Both populations upregulate calbindin expression right after they become postmitotic cells and as they leave the progenitor area. In the case of the Renshaw cells, they will extend lateral projections to the surface of the spinal cord in a first step and then once they reach the surface they will project ventrally and locate their cell bodies in between the motor pools and motor axon exit. At this time they upregulate MafB expression. The late generated V1-derived INs, which will include IaINs, are generated mostly between E10.5 and E12.0, with its maximum at E12.0. These cells upregulated the Foxp2 transcription factor immediately as the cells become postmitotic and take a more diagonal path towards their final positions settling just medial to the lateral motoneuron pools.

In conclusion, our study suggests that Renshaw cells and IaINs are differentiated early during embryonic development, exiting the cell cycle at different times, expressing different transcription factors and following distinct migratory pathways to reach their final locations.
CONCLUSIONS

In this thesis, we hypothesized that Renshaw cells and IaINs differentiate from each other early during embryonic development. We determined that:

1. Different classes of V1 INs have different birth dates. Renshaw cells are the earliest group and are born at the same time as motoneurons. V1-derived IaINs are generated later and through a much broader window time. There were no apparent differences in generation time between lower and upper lumbar segments.

2. Foxp2 and MafB are transcription factor that divide V1-INs into two non-overlapping populations. MafB is present in 100% of Renshaw cells, although is not exclusive of this cell type at P0. FoxP2 is expressed at P5 in several V1 and non-V1 ventral IN populations including 70% of V1-derived IaINs that we can recognize with histological criteria. Both transcription factors are expressed early during development, pointing to an early differentiation of both subtypes. MafB expression in Renshaw cells appears after the cells migrate and reach their final locations. In contrast, FoxP2 is upregulated in late-generated V1-INs before migration and as they become postmitotic and exit the progenitor area. Interestingly, calbindin is upregulated in early generated V1-INs, including Renshaw cells, as they become postmitotic and before migration. Calbindin expression is therefore an earlier marker of phenotypic differentiation in Renshaw cells than MafB.
3. V1-IN migration depends on time of exit from the cell cycle. Renshaw cells are generated very early and migrate in two steps, first straight from the progenitor area to the lateral edge of the spinal cord and then send projections to the ventral motor axon exit where they finally relocate their cell bodies. Later generated neurons follow a diagonal path towards the motor pools and stop medial to motor pools or in between them.

Overall our experiments support the idea that there are two main groups of V1-derived INs, early and late generated. The early generated V1-derived INs, which include among others Renshaw cells and big-calbindin-IR cells, are generated in between E9.5 and E10.5. Both populations upregulate calbindin expression right after they become postmitotic cells and as they leave the progenitor area. In the case of the Renshaw cells, they will migrate ventrally towards the ventral root region. When they reach their final position they upregulate MafB. The late generated V1-derived INs, which will include IaINs, are generated mostly between E10.5 and E12.0, with its maximum at E12.0. These cells express the transcription factor FoxP2 immediately after they become postmitotic and take a ventro-lateral diagonal path towards their final positions settling just medial and dorsal to the lateral motoneuron pools.

In conclusion, our study shows that Renshaw cells and IaINs are differentiated early during embryonic development, exit the cell cycle at different times, express different transcription factors and follow distinct migratory pathways to reach their final locations. Time of birth, transcription factor expression and migration all seem interrelated in directing the differentiation of these subclasses of V1-INs.
Figure 55. Summary of Renshaw cell and V1-derived IaIN differentiation. 

A) At E10.5 calbindin-IR V1-INs exit the progenitor area and extend projections, first laterally, then ventrally to the area in between motor pools and motor axon exit. 

B) At E11.5 differentiating Renshaw cells have migrated ventrally through the lateral edge of the spinal cord and position themselves at the motor axon exit region. A few upregulate MafB expression at this age but only once they have reached their final location. In addition, other calbindin-IR V1-INs are located more medially to motoneurons. Finally, there are new V1-INs exiting the progenitor area and these are calbindin negative and upregulate FoxP2 expression. 

C) At E12 more FoxP2 positive V1-INs leave the progenitor area and MafB is clearly expressed by all Renshaw cells. 

D) At E12.5 there are no more V1-INs added from the progenitor area. FoxP2 V1-INs finalize their migration and start to differentiate. They remain different from Renshaw cells and other calbindin-IR V1-INs. V1 cells have reached at this age their final position in relation to the motor pools. 

E) At P0/ P5, MafB and FoxP2 start to downregulate. MafB is present in calbindin-IR V1-INs that receive strong input from motor axons. FoxP2 is present in more dorsal V1-INs that receive inputs from Ia afferents and some can be defined as V1-derived IaINs. 

F) At P15 Renshaw cells and V1-derived IaINs have matured and Mafb and FoxP2 are downregulated.
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