Published in final edited form as:

Ann N Y Acad Sci. 2013 March ; 1279: . doi:10.1111/nyas.12084.

Principles of interneuron development learned from Renshaw cells and the motoneuron recurrent inhibitory circuit

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Abstract

Renshaw cells provide a convenient model to study spinal circuit development during the emergence of motor behaviors with the goal of capturing principles of interneuron specification and circuit construction. This work is facilitated by a long history of research that generated essential knowledge about the characteristics that define Renshaw cells and the recurrent inhibitory circuit they form with motoneurons. In this review, we summarize recent data on the specification of Renshaw cells and their connections. A major insight from these studies is that the basic Renshaw cell phenotype is specified before circuit assembly, a result of their early neurogenesis and migration. Connectivity is later added, constrained by their placement in the spinal cord. Finally, different rates of synapse proliferation alter the relative weights of different inputs on postnatal Renshaw cells. Based on this work some general principles on the integration of spinal interneurons in developing motor circuits are derived.

Keywords

Interneuron; spinal cord; neurogenesis; synaptogenesis; differentiation

Introduction: current context on interneuronal diversity and spinal circuit development

Spinal cord motor function depends on the wiring and properties of the interneurons that modulate motoneuron firing and motor output. A long-term objective is to gain a better understanding of the development and integration of interneurons in spinal circuits during maturation of motor function. One major challenge for understanding the principles of spinal cord circuit organization and construction is the large diversity of spinal interneurons.¹ Motoneuron to interneuron ratios in the lumbar 5 spinal segment of the newborn mouse are around 1:15 (Ref. 2), representing an intricacy in local circuits that is orders of magnitude higher than those in other parts of the central nervous system; in the cortex, for example, the average ratio of pyramidal cells to interneurons is 10:1.^{3, 4}

Basic questions then are, How many different types of interneurons exist, and what principal categories explain the function and development of spinal motor circuits. We cannot yet

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Conflicts of Interest

The authors declare no conflicts of interest.

answer these questions, but an overarching organizing principle is that all spinal interneurons throughout the vertebrate phylogeny derive from just 10 progenitor domains,^{5, 6} each generating an independent interneuron lineage (dl1–dl6 dorsally and V0–V3 ventrally). Each lineage diversifies into multiple subtypes but shares fundamental properties like laminar location, mediolateral positioning, and the primary direction of the axon (ascending, descending, ipsilateral, or contralateral). Mechanisms that create diversity within each canonical group are only now beginning to be understood. As reviewed later on, they start at the progenitor level; thus, adult interneuron phenotypes are specified before circuit assembly, challenging present definitions of interneuron classes based on adult connectivity and circuit function.⁷

Functional subtypes sometimes appear to be derived from a single lineage (e.g., Renshaw cells⁸), but this is not necessarily true for other functional classes, such as Ia inhibitory interneurons (IaINs) that have diverse origins.⁹ Thus, it is important to emphasize that physiological and molecular/developmental classifications group interneurons are based on different perspectives and distinguishing criteria. While physiology-based classifications take into account the inputs received by the interneuron and its action on target motoneurons, classifications based on molecular/genetic characteristics consider interneuronal derivation from particular progenitor domains and unique sets of gene coexpression directed by specific transcription factor combinations. These patterns of specific gene expression direct fundamental aspects of cellular development related to migration and placement, axonal projection type (e.g., commissural, ipsilateral, ascending, descending, short, long), and neurotransmitter phenotype. Because these features sometimes define the functional properties of mature neurons, we could expect, in some situations, a close relationship between a unique genetic/developmental subclass and a functional subgroup (e.g., Renshaw cells or cholinergic partition cells); however, in other situations, the correspondence might be not as clear. For example, a basic property that varies among different genetically defined groups is the orientation of the axon, implying that some functions, like reciprocal inhibition, may recruit interneurons from various classes according to the axon trajectories necessary to establish the required synaptic connections. To comprehend the fundamental categories of spinal interneurons, it is therefore necessary to understand the development of their basic properties and connections. A conceptual framework was laid out by the discovery of a few discrete progenitor domains; further work is now needed to understand the process of differentiating adult interneurons from these domains.

Another important difficulty is the changing nature of spinal interneurons, and their inputs, during embryonic and postnatal development. In mammalian species, major spinal inputs and outputs develop sequentially:^{10, 11} motor axons are first developed and connected to muscle around embryonic day 14 (E14);¹² proprioceptive afferents establish connections in the ventral spinal cord around E16;¹³ and finally, descending inputs arrive during late embryonic and early postnatal development (these dates always refer to development in the mouse lumbar spinal cord). The corticospinal tract is one of the last projection systems to form synapses in the lumbar cord, around postnatal day 6 (P6).¹⁴ In contrast, neurogenesis, differentiation, and migration of ventral interneurons are completed by E12, an age at which morphological or functional evidence for synapses is scarce.^{15–17} Thus, ventral interneurons are specified, and start differentiation in a largely synapse-free environment. Lack of synapses does not mean that there is no active neurochemical communication among the cellular elements of the earliest spinal network. In fact, E11–E12 mouse spinal cords display spontaneous activity driven by cholinergic, GABAergic, and glycinergic signaling,^{18, 19} which could rely on the few synapses found at this age, but are more likely paracrine in nature¹⁷ and/or based on electrical coupling. Within this environment of relatively nonspecific neurochemical interactions, synaptic inputs are sequentially added onto specific

cellular elements, and this process likely changes the character and function of developing interneurons from birth to circuit maturation.

Our approach is to study a few spinal circuits and interneurons whose adult characteristics and variety are well known. We focus on the final spinal cord output module that consists of motoneurons synaptically coupled to Renshaw cells and IaINs, which respectively exert recurrent and reciprocal inhibition of motoneurons (Fig. 1). Here, we review what we know (or don't know) about Renshaw cells and the recurrent inhibitory circuit they form, making some comparisons to the sparse information available on IaIN development.

Basic properties of recurrent inhibition and Renshaw cells

Renshaw cells and motoneurons form a recurrent inhibitory circuit that controls motor output. Individual Renshaw cells receive inputs from particular motor pools and spread their inhibitory output to the same motoneurons, their synergists (i.e., motor pools exerting a similar action on the same joint), and sometimes selected motor pools across joints.^{20–23} Renshaw cells are located in the most ventral regions of LVII and LIX, defined since 1965 as the *Renshaw cell area*,²⁴ and send axons into the adjacent ventral funiculus. In the mature spinal cord, Renshaw axons bifurcate, sending both ascending and descending branches, from which collaterals enter lamina IX and primarily target motoneuron proximal dendrites.²⁵ Renshaw cells are thus designed to monitor motor output and exert feedback modulation of excitatory inputs, such as Ia afferent inputs, to motoneuron dendrites, as first reported by Renshaw in 1941.²⁰

The principal distinguishing feature of Renshaw cells is that they are the main intraspinal target of motor axons. In addition, Renshaw cells exhibit other properties, like calbindin immunoreactivity, that distinguish them from other interneurons (Table 1; see also Ref. 27 for more detailed descriptions and references). Together, they construct interneurons that, in the adult, integrate incoming motor axon synaptic inputs to their dendrites²⁷ and are strongly modulated by a high density of large inhibitory synapses located more proximally.^{28–30} The excitatory postsynaptic potentials (EPSPs) evoked by motor axons on Renshaw cells are of longer duration than on muscle, in part because of the incorporation of slow NMDA components in the Renshaw cell response in addition to the faster nicotinic responses.^{31–34} Renshaw cell output is characterized by burst firing that outlasts the synaptic input, at least in the typical recording conditions of classical experiments (with pentobarbital anesthetized cats),^{21–23} and in more recent *in vitro* preparations, like the isolated spinal cord and transverse spinal cord slices from mice. Their inhibitory synapses on motoneurons are designed to further lengthen inhibitory synaptic action by addition of GABAergic components that have slower time courses than pure inhibitory glycinergic neurotransmission³⁵ (see also Ref. 36 for more recent data). Temporal summation of successive slow IPSPs during burst firing explains the typical compound recurrent IPSP recorded after a ventral root volley, and consisting of a slow “Renshaw ripple” in the rising phase and long decays.²² The combination of these cellular properties produces recurrent IPSPs that are of longer duration than the more phasic reciprocal inhibition elicited by IaINs.

Expression of calbindin, a calcium-buffering protein, is a distinguishing feature of Renshaw cells that is abundant in their axons and dendrites.²⁶ Its function has not been investigated directly, but the BAPTA-like calcium-buffering properties of calbindin, and its presence throughout the Renshaw cell axon and synaptic terminals, could facilitate summation of successive release events in a synaptic train, as occurs in other calbindin-expressing interneurons.³⁷ On the other hand, dendritic calbindin is well positioned to modulate calcium transients induced by opening calcium-permeable nicotinic and NMDA receptors associated with motor axon synapses.³³ In summary, Renshaw cells are endowed with

cellular properties (Table 1) that give them quite distinct functional characteristics. An important question then is, How do these properties develop to construct the mature Renshaw phenotype.

Lineage origins of Renshaw cells and temporal control of cell phenotype

It has been known for some time that Renshaw cells originate from p1 progenitors and therefore belong to the V1 interneuron subclass.⁸ They share with other V1s the early expression of the transcription factor, engrailed-1, an inhibitory phenotype, and extension of ipsilateral ascending axons. However, within this class they constitute just under 10% of all V1s in the mouse lumbar cord.³⁸ How is their phenotype specified within the V1 class?

Several mechanisms can create interneuron diversity from single progenitor domains. In some instances, daughter cells are differentially fated through asymmetric last divisions, as for example the diversification of p2-derived interneurons into excitatory V2a and inhibitory V2b cells.^{39–41} In other cases, different lineages are created through spatial and/or temporal heterogeneity within each progenitor domain, as occurs during specification of subclasses of V0 commissural interneurons from distinct groups of p0 progenitors.^{42, 43} Remarkably, these mechanisms are conserved from zebrafish to mice, but the phenotypic variation generated from each domain is much larger in mice, as should be expected, given the larger complexities of limbed terrestrial motor function. While, in zebrafish and tadpoles, p1 progenitors generate one type of interneuron of well-defined morphology and function during swimming,^{44, 45} a recent preliminary report divides V1 interneurons in the neonatal mouse lumbar cord into over half a dozen populations, each defined by overlapping expression of 18 transcription factors.⁴⁶

Renshaw cell specification from the V1 lineage is based on temporal control of their neurogenesis.⁴⁷ V1 interneurons can be divided into early- and late-generated groups defined by the time they exit the progenitor cell cycle and start differentiation. These groups are further distinguished by expression of the transcription factor, Forkhead box protein P2 (FoxP2). Early-born V1s start differentiation between E9.5 and E10.5, and lack FoxP2 expression; many upregulate calbindin soon after being generated. Within this group, Renshaw cells constitute a homogenous cohort generated in a narrow temporal window at the beginning of V1 neurogenesis.^{47, 48} Thus, in the E10–10.5 lumbar cord, most differentiating V1s are calbindin Renshaw cell precursors. In contrast, late-born V1s exit the progenitor cell cycle between E11 and E12, rapidly upregulate FoxP2, and do not express calbindin. Interestingly, some FoxP2 V1s display synaptic connectivity in the postnatal spinal cord that resembles that of IaINs, and some upregulate a different calcium binding protein (parvalbumin) in the second postnatal week.^{9, 47} Temporal control of neurogenesis is currently considered a primary mechanism, inducing ventral interneuron diversity,⁴⁹ as is the case for the V1 lineage.

A yet unresolved question is whether the early generation of Renshaw cells is due to derivation from an early p1 pool of progenitor cells that then becomes exhausted or if, on the contrary, Renshaw cells are clonally related to later V1s. A clonal analysis of V1 phenotypes derived from single p1 progenitors will be necessary to resolve this issue. The first possibility might advocate for a certain genetic uniqueness of Renshaw cells. In any case, early genesis confers Renshaw cells with properties that influence their differentiation and integration into specific synaptic circuitry. First, early generation is related to a specific transcriptional code, inducing a *Renshaw cell genetic program*.⁴⁸ Second, early genesis permits Renshaw precursors to enter a migratory pathway that places them in a close relationship with motor axons,⁴⁷ and finally, early V1s are specifically associated with calbindin expression.⁴⁷

A Renshaw cell-specific transcriptional code and calbindin expression

Recent work has described several transcription factors involved in Renshaw cell differentiation⁴⁸ and whose expression is specifically associated with the earliest interneurons generated from p1 progenitors. Two key transcription factors are Forkhead box protein d3 (Foxd3) and v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MafB). Both are also expressed by other V1 and non-V1 interneurons, being that the timing and length of expression are important factors in Renshaw cell specification. Foxd3 is broadly, and transiently, expressed by V1s just after birth, while Renshaw cells maintain Foxd3 expression for a longer period. Deletion of Foxd3 prevents Renshaw cells from expressing calbindin and MafB, and from forming a recurrent inhibitory circuit. MafB is expressed later and its deletion results in calbindin downregulation in differentiated Renshaw cells.⁴⁸ Foxd3 and MafB are therefore related to expression of calbindin in Renshaw cells. Other early-generated V1s also transiently express calbindin,⁴⁷ but while calbindin is downregulated in most V1s during development, mature Renshaw cells retain strong expression.⁹ The previously mentioned results can thus be interpreted as Foxd3 being involved in induction of a calbindin V1 phenotype (and perhaps also other Renshaw properties), and its prolonged expression, together with MafB, acting to consolidate calbindin expression and the Renshaw phenotype.

Renshaw cell circumferential migration and placement at the ventral root exit

At the time when Renshaw cells are born, there are few cellular elements in the ventral horn other than motoneurons, which are generated at times overlapping with Renshaw cells. This early specification promotes a close relationship between motor axons and Renshaw cells because of the availability of a migratory pathway toward the ventral root that is taken by the early cohort of V1s (Fig. 2). The scarcity of cells in the mantle layer during the time Renshaw cell precursors originate implies that these precursors are rapidly placed at the lateral border of the embryonic cord, above motor pools. Soon after, they extend ventrally oriented neurites that surround the motor pools circling the lateral edge of the embryonic ventral horn, and end in a growth cone that stops at the ventral root exit. Later, their cell bodies translocate following the path established by these neurites. The end result is that differentiating Renshaw cells are distributed among exiting motor axons. Later generated V1s do not display this circumferential migration and accumulate medially to motoneurons, thus distal to motor axons.⁴⁷

These findings suggest that placement among exiting motor axons early in development is a critical step that defines the preferential targeting of Renshaw cells by motoneurons. For this to occur, two mechanisms are necessary: first, Renshaw cells need to migrate toward the ventral root exit; second, motor axons must be prevented from spreading outside the ventral root area. Although the signals that control these processes are unknown, clearly both mechanisms occur (Fig. 2) and there are interesting parallels in other spinal systems. For example, placement was identified as the major principle predicting connectivity among interneurons and motoneurons in tadpole spinal cords,^{50, 51} and recent elegant studies showed that positional cues are a major influence restricting monosynaptic Ia afferent connections to the correct motor pools.^{52, 53} Recent molecular studies also suggest that this process is based on constraining the trajectories of different inputs while placing specific cellular targets in their paths. Thus, Ia afferent axon trajectories are controlled by semaphorin/plexin interactions,⁵⁴ while motor pool placement is influenced by the transcription factor, FoxP1, perhaps by regulating patterns of cadherin-catenin signaling, which directly control topographic order and motor pool positions.^{53, 55}

In conclusion, the mechanisms that define early cellular placement seem to have an important influence on the major inputs that different cells will receive later. This is because, as shown for Ia afferent trajectories and the extension of motor axon collaterals in embryos, different axonal systems are allowed to course only through specific regions of the ventral horn, each containing specific neural targets. Such topographic relationships establish an early connectivity map that can be later refined through other mechanisms, including activity-dependent synaptic strengthening, or weakening, of specific inputs.

Early synaptogenesis and formation of the recurrent inhibitory circuit

Most pre- and postsynaptic markers of excitatory and inhibitory synapses are upregulated in the mouse spinal cord at E13 or later, and this corresponds with a large increase in the number of synapses detected by electron microscopy.^{15, 16} Before synaptogenesis, synaptic proteins, including those related to synaptic vesicles, are diffusely distributed in axons, but at around E13, vesicular acetylcholine transporter (VACHT) immunoreactive motor axons establish a close relationship with calbindin-IR Renshaw cell bodies and dendrites (Fig. 2), providing the first evidence of putative synapses in the ventral horn. Interestingly, at E13, there is no anatomical evidence for synaptic connections from Renshaw cells onto motoneurons. Renshaw cell axons are first found in the ventral funiculus at E11, immediately after Renshaw cell migration is completed. From E11 to E13, they extend for a few segments rostrally without forming collateral branches entering lamina IX. The earliest evidence for calbindin synaptic collaterals innervating lumbar motoneurons was found at E15, and these synapses increase in frequency by E17 (Fig. 2). By P4, a secondary descending branch has formed that innervates motoneurons caudal to the Renshaw cell body location.⁵⁶ It is noteworthy that, in the chick embryonic spinal cord, the spread of recurrent inhibition has been shown to undergo refinement during embryonic development; however, the exact mechanisms have not yet been directly investigated.⁵⁷

Although many details still need elucidation, one conclusion is clear: the recurrent inhibitory circuit is formed in two steps. First, motor axons synapse onto Renshaw cells and, later, Renshaw axons synapse onto motoneurons. The significance of this chronological order is unknown, but one possibility is that Renshaw axons, similar to thalamocortical axons,⁵⁸ require a waiting period until their targets mature certain properties (importantly, their final spatial distributions, see Fig. 2). This sequence of events does not imply that Renshaw differentiation requires early motor axon synaptic input. Rather, Renshaw cells differentiate in normal numbers and locations in choline acetyltransferase knockouts¹⁹ and recurrent inhibitory circuit connections form normally in the absence of synaptic activity.⁴⁸ Thus, activity is dispensable during early circuit formation but influences later circuit maturation.

Late addition of Ia afferent inputs

As mentioned previously, different inputs arrive at the spinal cord sequentially, and one set of inputs that reaches the Renshaw area relatively late are Ia afferent axons. Ia afferent synapses are formed on a few Renshaw cells around E16–E17. At birth, most Renshaw cells receive Ia afferent inputs capable of evoke firing. By P10, they have spread and innervate all Renshaw cells in lumbar segments.⁵⁶ The presence of these synapses was surprising because adult Renshaw cells are believed to lack Ia afferent input.^{7, 31} Its functional significance during development is yet unknown, but it does not imply that Renshaw cells share circuit functions with IaINs in newborns. Renshaw cells receive fewer Ia afferent synapses than do IaINs in neonates, and it is unlikely that Ia inputs on Renshaw cells are organized in reciprocal circuits. Following the idea of positional specification of sensory afferent inputs, it follows that the origins and strength of Ia inputs is likely influenced by V1 interneuron location. Thus, IaINs are distributed throughout the dorsoventral extent of LVII and receive

a higher density of Ia afferent vesicular glutamate transporter 1 (VGLUT1) synapses than the more ventrally located Renshaw cells.⁹ Moreover, pools of IaINs located at different positions receive innervation from Ia afferents originating from different muscles.^{59, 60} This cannot be the case for Renshaw cells, which are all clustered in the most ventral region of the ventral horn, an area traversed by Ia afferents from just a few muscle groups.⁶¹ Given the large input resistance of Renshaw cells at P4, dorsal root stimulation elicits robust Renshaw responses;⁵⁶ however, Ia afferent firing might not be very active in neonates since Ia afferent stretch responses are still maturing.⁶² As reviewed subsequently, this input weakens in Renshaw cells during later circuit maturation while being strengthened in IaINs. Thus, all VIs seem permissive for receiving Ia afferent synapses, but the initial Ia input strengths (and maybe their organization) are related to cell location. During later maturation, Ia inputs become strengthened or deselected in different VIs.

Maturation of synaptic numbers and input selection

There is a significant addition of synapses in the mouse spinal cord during the first two postnatal weeks, a period that also coincides with the development of weight-bearing locomotion, reflex maturation, and improvement of motor coordination. Correspondingly, both Ia afferent and motor axon synapses proliferate on Renshaw cells from P0 to P15; however, while motor synaptic density on Renshaw dendrites is maintained after P15, Ia afferent synaptic density decreases.^{9, 56} This decrease is not the result of active synapse withdrawal, but of arrested proliferation creating mismatches with dendritic growth. Ia synapses on mature Renshaw cells also display decreased synaptic complex sizes⁵⁶ and AMPA receptor content (Francisco J. Alvarez, unpublished observations). In contrast, Ia synaptic density is maintained on IaINs during the same period.⁹ Interestingly, the reduction of Ia afferent synaptic density on Renshaw cells is prevented in a mouse model with postnatal muscle overexpression of neurotrophin-3 driven by the myosin light chain promoter (see Fig. 3 in Ref. 63), a situation that increases Ia input strength.⁶⁴ Therefore, postnatal maturation of synaptic numbers seems to be an activity-dependent process that shifts the relative weights of different inputs on Renshaw firing. Mature Renshaw cells are driven to fire by motor axon synapses and not by Ia afferents. During the same period, inhibitory synaptic strength is adjusted by the amount of excitation that Renshaw cells receive from motor axons,²⁹ a process that could further decrease the influence of weaker excitatory inputs.

In summary, postnatal regulation of synapse numbers reconfigures Renshaw cell synaptic integrative properties by strengthening some inputs and weakening/silencing others within the connectivity plan laid down in embryo. This tuning is also affected by dendritic growth,⁵⁶ developmental regulation of inhibition,²⁹ alterations in neurotransmitter receptor subunit compositions,²⁸ and expression of different voltage-dependent channels (for example, faster KV3 channels upregulate in late postnatal Renshaw cells).⁶⁵ This process implies that perhaps not all anatomical connectivity is functionally expressed in normal conditions in adult spinal networks, an idea that has also recently been proposed in the tadpole spinal cord.⁵¹

Conclusions

Although many details are still missing, the developmental model of Renshaw cells provides, for the first time, a view of interneuronal development that is sufficient for a preliminary description of several discrete phases (Fig. 3). First, interneuron specification occurs during neurogenesis on the basis of the induction of specific genetic programs at the progenitor level. These programs control axonal trajectories, neurochemical phenotypes, migration and spinal cord placement, and are determinants of later circuit assembly. Second,

a connectivity blueprint is established on the basis of positional information, such that growing axons are channeled along pathways that cross locations containing specific interneuron targets. Third, initial synaptogenesis occurs among neighboring axons and dendrites through mechanisms that are independent of activity. Given the high excitability of embryonic interneurons, most synapses are initially capable of evoking postsynaptic firing in embryonic and early postnatal interneurons. Fourth, later postnatal synapse proliferation and maturation is dependent on activity, and occurs in conjunction with maturation of other postsynaptic properties that influence the synaptic integrative capabilities and excitability of interneurons. Postnatal maturation selects from the previously formed connectome the inputs that will exert major influence on firing activity of adult interneurons. This final phase results in mature circuits capable of producing adult coordinated motor output.

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Figure 1.

Diagram of basic connectivity in the recurrent and reciprocal inhibitory circuit controlling motor output, superimposed on a Nissl stained section of the lumbar spinal cord. Motoneurons are arranged in pools that innervate different muscles. While Renshaw cells receive inputs from certain pools and provide feedback inhibition to the same motoneurons and its synergists, Ia inhibitory interneurons mediate reciprocal inhibition, such that they inhibit motor pools with antagonist actions to the muscle of origin of the Ia afferent, thus permitting smooth flexor extension around individual joints. Ia inhibitory interneurons and motoneurons receiving common Ia inputs also receive similar excitatory drive from other systems, such that activation of motor pools is always coupled with relaxation of antagonists. However, Ia inhibitory interneuron activation is tightly controlled, in part, by the Renshaw cells themselves, so that the amount of relaxation or cocontraction of antagonist muscles is finely modulated. Excitatory drive onto motoneurons acts on this last order circuit involved in the last step of motor control. Each of the two interneurons display specific placement in the ventral horn and in connectivity with target motoneurons and incoming Ia afferents, as depicted in the diagram.

Figure 2.

Early formation of the recurrent inhibitory circuit in mouse embryos. In E10.5 embryos, Renshaw cell precursors (RCs) are identified as the first calbindin (CB)-IR interneurons generated in the spinal cord. Other CB-expressing cells are present in the floor plate (FP) and dorsal root ganglion (DRG). This E10.5 section was counterstained with Tuj1 antibodies to depict the location of differentiating neurons and their axons. At this age, ventral roots are already formed and motor axons are entering the limb buds. CB-IR Renshaw cells are exiting the progenitor zone (negative for Tuj1 immunoreactivity) and position themselves laterally. By E11.5, two populations of CB-IR interneurons are present in the ventral horn. Most CB-IR interneurons in central regions are not RCs; they correspond with interneurons that, in their majority, will downregulate CB during embryonic and early postnatal development. RC precursors are located at the lateral edge and actively migrate toward the ventral root. This pathway surrounds the motoneurons (labeled by EGFP, driven by the Hb9 promoter), which have not yet started to separate into discrete columns (although they already express different EGFP levels). At this time, the ventral funiculus is formed (axons heavily immunoreactive for SV2) and CB-IR RC axons appear for the first time at this location. Between E12 and E13, the major motor columns of the lumbar spinal cord (MMC, LMCd, LMCv) can be distinguished by different levels of EGFP expression and they start to segregate spatially. During this time, we find the first evidence of immunoreactivity against the vesicular acetylcholine transporter (VACHT). VACHT-IR axons are restricted to locations containing motor pools, motor axons, and RCs. The first evidence of EGFP and VACHT-IR processes in contact with RCs (arrows) is also apparent at this time, suggesting the possibility of early motor axon synaptic interactions. RCs emit an ascending axon located in the ventral funiculus, but this has no synaptic collaterals entering the spinal cord. A large invasion of the motor pools by synaptophysin-containing axons does not occur until E15. Before that time, all synaptic markers are restricted to the developing white matter. The presence of synaptophysin processes coincides with the presence of CB-IR axons putatively originating from RCs around motoneurons. It is also at this time that the spatial relationships of different motor columns mature and a tight cluster of RCs is formed between the LMC and MMC in front of the ventral root, now placed more ventrally instead of more laterally,

due to spinal cord morphogenetic maturation. Some CB-IR processes are in contact with EGFP-labeled motoneurons and contain synaptophysin, suggesting the presence of synapses. Finally, other inputs, like Ia afferents, are added later. In the image, dorsal root (DR) axons were anterogradely labeled by filling the dorsal roots with FITC-conjugated dextrans in a section that was also immunostained for CB. CB-IR RCs are at the very bottom of the ventral horn, which explains the relatively late formation of Ia afferent synapses on them.

Figure 3.

Landmark steps in the specification of Renshaw cells and their connections (events above the time line) in the context of other developmental processes in the spinal cord (events below the time line), and the corresponding motor output. Dates are approximations because of differences associated with spinal cord rostrocaudal level and even small differences within litters, with embryos showing more or less advanced maturation.

Table 1

Major features that characterize the Renshaw cell phenotype

Property	Distinguishing features
Cell body location	Ventral most LVII and LIX in front of ventral root exit
Axon	Ipsilateral, ventral funiculus
Characteristic input	Motor axons/nicotinic cholinoreceptive neurons
Characteristic output	Homonymous motoneurons and synergists
Firing properties	Burst firing to motor axon inputs in anesthetized animals
Neurotransmitter phenotype	Glycine and GABA
Calcium regulation	Strong expression of calbindin calcium-buffering protein
Synaptic architecture	Large inhibitory synapses on cell body and proximal dendrites, and segregation of excitatory synapses to more distal dendrites