Neonatal Circuits

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Introduction

Rhythmic patterns of coordinated movement are produced by networks of spinal neurons known as 'central pattern generators' (CPGs). These circuits have been studied using isolated spinal cord preparations that can generate a pattern of motor discharge that resembles locomotion (locomotor-like activity). The isolated spinal cord is more experimentally accessible than the equivalent *in vivo* preparation and allows network function to be studied under controlled ionic and pharmacological conditions. For technical reasons, studies of central pattern generation in isolated preparations are restricted to the neonatal period and cannot be performed on adult mammals. In this article, we first consider the general properties of the locomotor-like rhythm in the neonatal rat and mouse cords and then discuss our knowledge of the specific circuitry responsible for this behavior.

Segmental and Spatial Distribution of the Pattern-Generating Circuitry in Neonatal Spinal Cord

The hind limb locomotor CPGs of the neonatal rat are distributed along the lumbar cord with a rostrocaudal decline in the rhythmogenic capacity of each segment. In the rat and mouse, rhythmogenic activity can also be observed in isolated sacrocaudal segments (Figure 1), indicating that rhythmogenic circuitry is not unique to the limb moving segments. The sacrocaudal network controls the axial and tail musculature during locomotor and other movements. Experimental activation of this network produces alternating left/right rhythmic bursts in sacral motor neurons. The thoracolumbar and sacrocaudal networks are coupled in the rostrocaudal direction via propriospinal/descending neurons, the axons of which travel in the lateral, ventral, and ventrolateral funiculi. These connections coordinate activation of the limb, axial, and tail musculature during movement.

It has been proposed that the locomotor rhythm is generated by antagonistic 'half-centers' activated by common tonic excitation and linked by reciprocal inhibitory pathways. Consistent with this idea, intracellular recordings from lumbar motor neurons in the neonatal rat show that the locomotor drive potentials produced in lumbar motor neurons during rhythmic activity induced by bath application of *N*-methyl-D-aspartate (NMDA) and 5-hydroxytryptamine (5-HT; serotonin) are generated by an alternating sequence of phasic excitation and inhibition. However, several findings argue against the half-center model as a mechanism for spinal rhythmogenesis. For example, the locomotor-like rhythm can be generated in isolated hemicords that necessarily lack contralateral inhibitory connections. Furthermore, studies of sacrocaudal networks in the neonatal rat spinal cord have shown that reciprocal inhibitory pathways between flexor and extensor motor neurons are not activated during the sacrocaudal rhythm. As a result, flexor, extensor, and abductor tail muscles are coactive on one side of the tail during rhythmic activation of the sacrocaudal networks (Figure 1(c)). Because the sacrocaudal rhythm persists after separating the cord midsagittally, it is clear that this rhythm does not require activation of either crossed or reciprocal synaptic inhibition (Figure 1(d)).

It is now believed that the function of the contralateral inhibitory and excitatory connections is to regulate the timing of motor neuron activation. Because contralateral motor neurons alternate during locomotion it is assumed that the contralateral inhibitory connections are substantially more powerful than are the excitatory connections during normal locomotion. However, an increase in the excitatory crossed connections – such as occurs in certain mutant mice or as in the presence of inhibitory amino acid receptor blockers – can change the contralateral motor neuron activation from alternation to synchrony (see later).

Sensory Pathway Interneurons and Pattern Generation in Neonatal Spinal Cord

Studies of Lundberg and colleagues have revealed that segmental pathways activated by high-threshold flexor reflex afferents (FRAs) produced a locomotor-like alternating flexor–extensor discharge in cats subjected to spinal cord transection ('spinal cats') treated with L-3,4-dihydroxyphenylalanine (L-DOPA) and monoamine oxidase blockers. It has been suggested that these FRA pathways are actually part of the locomotor pattern generators, because stimulation of FRA components can reset the L-DOPA-induced rhythm. In addition, application of opioids to spinal

cats can block the spontaneous locomotor-like rhythm induced by L-DOPA and the alternating flexor–extensor pattern produced by stimulation of various FRA components.

Alternatively, FRA interneurons may be interposed between the locomotor CPGs and their target motor neurons. Studies of the sacrocaudal network in the neonatal rat and mouse have revealed that low- and high-intensity stimulation of sacrocaudal afferents (SCAs) can produce a coordinated activation of the locomotor and sacrocaudal CPGs (Figure 2(a)). The high accessibility of the sacrocaudal network and the extraordinary capacity of SCA pathways to produce rhythmic patterns in the lumbar and sacral cord have been recently exploited to study the role of the pathway interneurons in pattern generation.

This work has revealed that the SCA-induced locomotor rhythm involves synaptic activation of relay neurons in the sacrocaudal cord. It was found that the SCA-induced locomotor rhythm could be

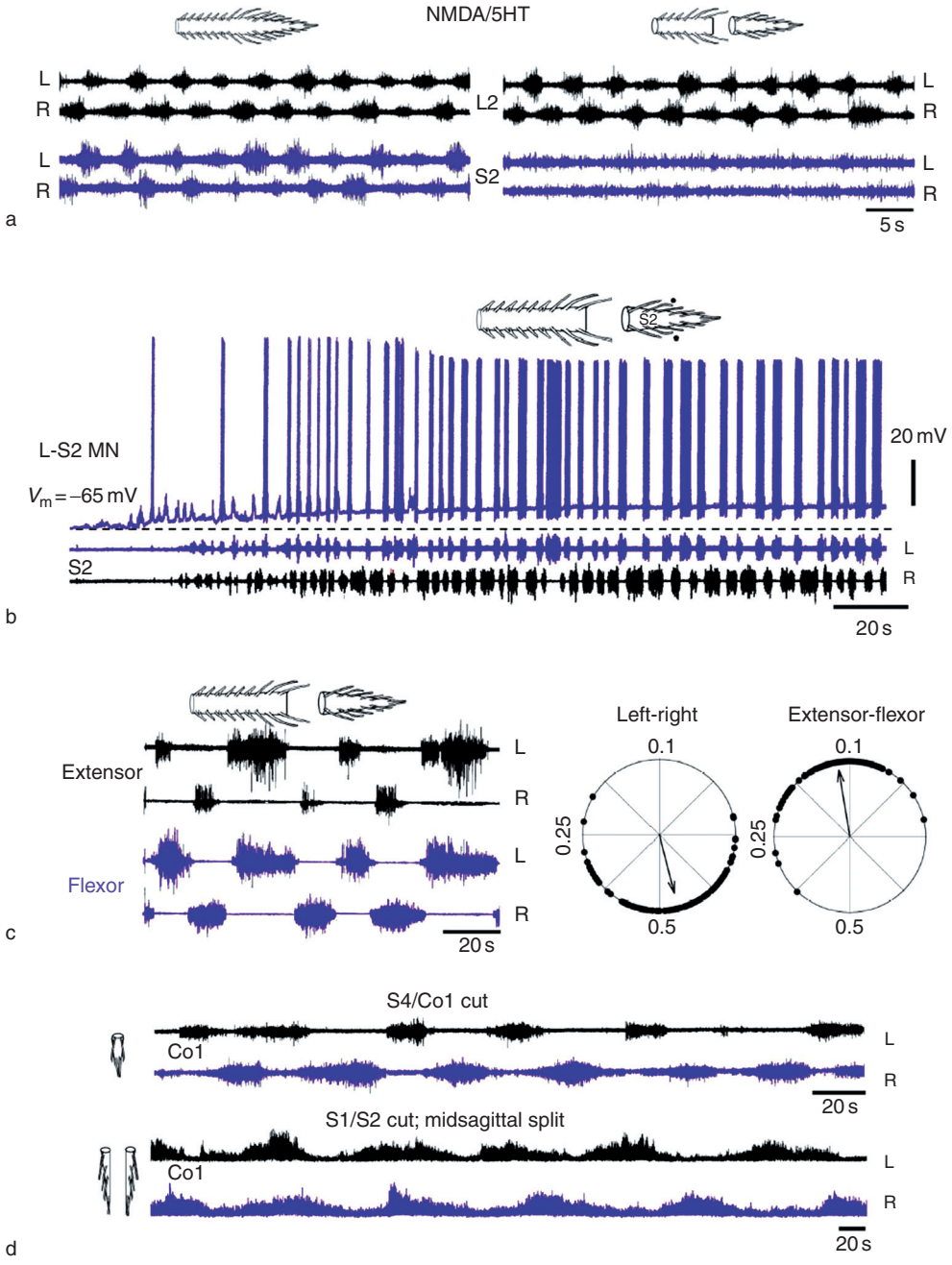

Figure 1 Continued

abolished after blocking synaptic transmission in the sacrocaudal segments by low-calcium, high-magnesium solution (Figure 2(b)). Moreover, specific application of μ-opioid receptor agonists to the sacrocaudal segments blocks SCA-induced rhythmic activity in the cord, although the rhythmogenic capacity of these networks is not impaired. This is because they can still be activated by bath application of NMDA/5-HT or by stimulation of the ventromedial medulla in the presence of μ-opioid receptor agonists. Physiological studies have shown that the axons of the majority of the sacrocaudal relay neurons activated by SCAs cross the spinal cord at the sacrocaudal level and ascend rostrally to the lumbar CPG mainly through the ventral funiculi (Figures 3(a) and 3(b)). More recent anatomical and physiological studies have indeed identified groups of sacrocaudal interneurons in the ventral horn, the intermediate zone of the gray matter, and the ventral parts of the dorsal horn (Figure 3(c)). These interneurons are innervated by SCAs and project their axons rostrally through the contralateral ventral funiculus. SCA stimulation activates these interneurons, an effect that is blocked by μ-opioid receptor agonists and which recovers in the presence of naloxone. It is suggested that these sensory pathway interneurons do not participate directly in the genesis of locomotion, and further studies are required to determine whether they contribute to sacrocaudal rhythmogenesis.

Specific Interneuronal Circuits

To understand the operation of locomotor networks requires identification of the constituent interneurons, their synaptic connections, and their intrinsic properties. In adult animals, spinal interneurons are generally defined by their synaptic connections. Such characterization is more difficult in the neonate because the connections may change during development and because the inputs and outputs of developing interneurons are frequently inaccessible. For these reasons, researchers have sought to identify subsets of developing neurons by their expression of various genetic markers. Among the most useful of these are combinations of transcription factors – proteins that regulate the expression of several genes. The interneuronal classes that have been identified using this approach contain the precursors of several different adult interneuron types.

We first consider the development of two types of interneurons that have been characterized in detail in the adult cat: the Renshaw cell and the 1a inhibitory interneuron. Both of these neurons have been shown to be rhythmically active during adult locomotion and are presumed to play an important role in locomotor activity. Renshaw cells, first described in cat in the 1940s, are the only known synaptic targets of motor neurons within the spinal cord. The 1a inhibitory interneurons receive direct inputs from primary muscle spindle afferents and project monosynaptically to antagonist motor neurons. Despite these differences, Renshaw cells and 1a inhibitory interneurons share many aspects of their connectivity. For example, they are each reciprocally interconnected among their own class and between each other (Renshaw–1a interneuron) and they both project monosynaptically to motor neurons.

In the neonatal mouse, shortly after birth, it has been possible to record activity from interneurons that receive direct monosynaptic connections from motor

Figure 1 Pattern generation in the isolated spinal cord of the neonatal rat. (a) Bath application of *N*-methyl-D-aspartate (NMDA) and 5-hydroxytryptamine (5HT) produces coordinated rhythmic activity in the lumbar (L2) and sacrocaudal (S2) segments of the spinal cord. The sacrocaudal rhythmicity is virtually blocked while the lumbar rhythm persists following transection of the cord at the lumbosacral junction. Recordings were obtained from the left (L) and right (R) ventral roots of L2 and S2 in preparations isolated from postnatal day 3 pups. (b) The α1 adrenoceptor agonist methoxamine (100 μM) produces rhythmic activity in the isolated sacrocaudal (S2) segments of the neonatal rat spinal cord. Intracellular recordings from a left S2 motor neuron (L-S2 MN; upper record, blue) show the development of tonic depolarization and superimposed sub- and then suprathreshold membrane potential oscillations in the presence of methoxamine. Ventral root population recordings from the left (L; blue) and right (R; black) S2 ventral roots are shown below the intracellular trace. (c) The sacrocaudal rhythmicity does not depend on activation of reciprocal inhibitory pathways between flexor and extensor centers. Electromyelogram recordings from left (L) and right (R) flexor and extensor muscles of the tail are shown following transection of the cord at the lumbosacral junction. The rhythmic pattern is characterized by alternating left/right activation of the tail muscles and by coactivation of flexor and extensor muscles within a given side of the tail. This flexor–extensor synchronicity indicates that reciprocal inhibitory pathways are not activated during the rhythm. The rhythm was induced by 10 μM noradrenaline and 3 μM NMDA. The circular diagrams (right) show the phase relation between the activities of the left and right muscles and between those of the ipsilateral extensor and flexor muscles during the rhythm, in five different experiments. (d)The sacrocaudal rhythm does not depend on activation of crossed inhibitory pathways between the left and right hemicords. Recordings from left and right ventral roots of Co1 in the isolated coccygeal cord (S4/Co1 cut) and in a midsagittally split S2/Co3 preparation (S1/S2 cut, midsagittal split; different preparation; data are rectified and integrated). Rhythmic activity was induced by 5 μM noradrenaline and 4 μM NMDA. (a) From Strauss I and Lev-Tov A, unpublished data. (b) Adapted from Gabbay H and Lev-Tov A (2004) Alpha-1 adrenoceptor agonists generate a 'fast' NMDA-receptor independent motor rhythm in the neonatal rat spinal cord. *Journal of Neurophysiology* 92: 997–1010. (c, d) Adapted from Gabbay H, Delvolvé I, and Lev-Tov A (2002) Pattern generation in caudal-lumbar and sacrococcygeal segments of the neonatal rat spinal cord. *Journal of Neurophysiology* 88: 732–739.

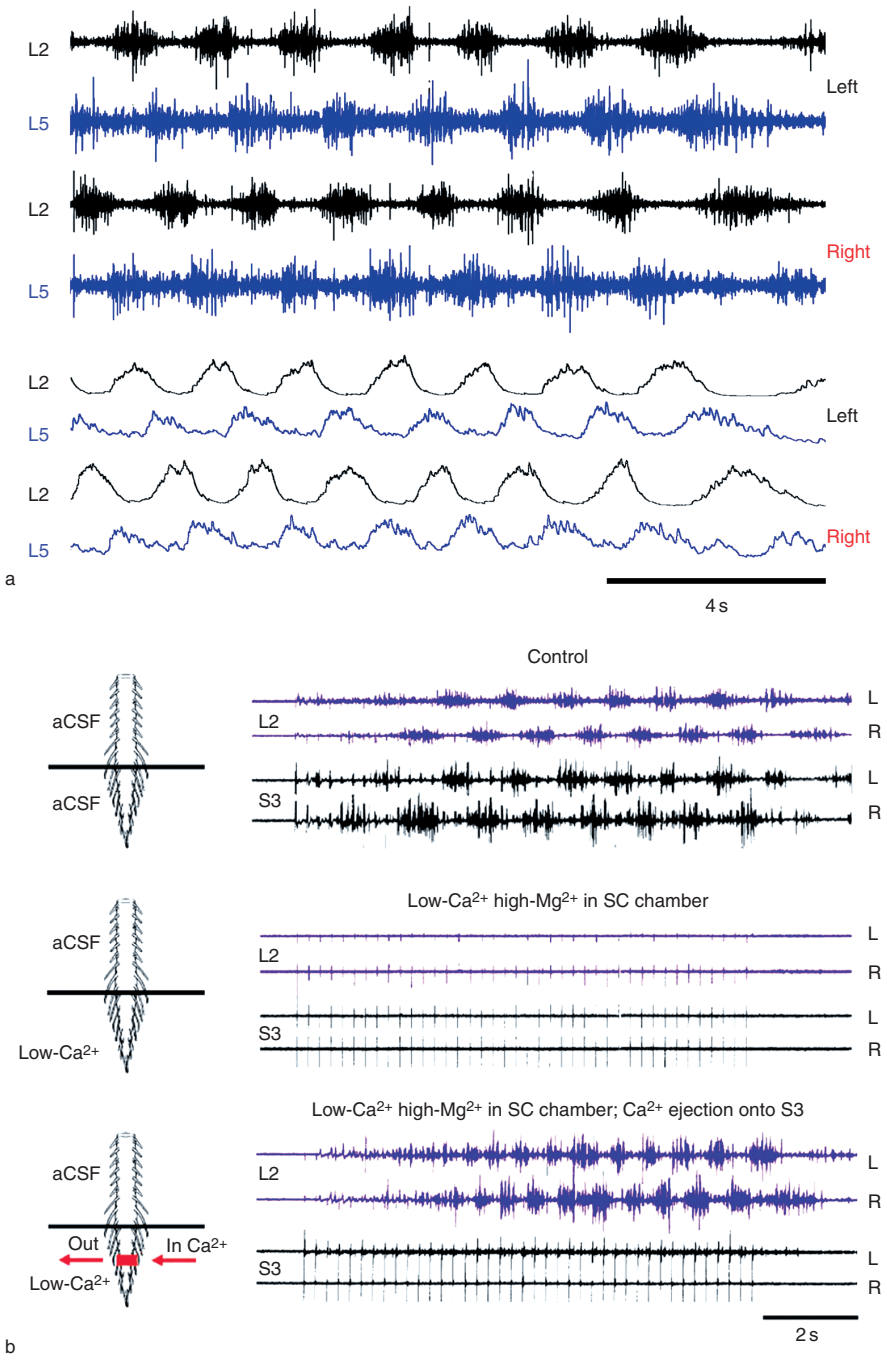

Figure 2 Sensory activation of the locomotor rhythm. (a) Recordings from the left and right ventral roots of the flexor dominating L2 (black) spinal segments and the extensor dominating L5 (blue) segments are shown during a stimulus train (50 pulse, 3 Hz) applied to the left coccygeal Co1 dorsal root at 1.8 T threshold. Upper set of recordings, 50 Hz–5 KHz; lower set of recordings, the same data following rectification and low-pass filtering at 10 Hz. Note the typical left/right alternating pattern as well as the ipsilateral alternation of flexor and extensor bursts. (b) The sacrocaudal afferent-induced locomotor rhythm is mediated by synaptic activation of sacrocaudal interneurons. Recordings of the activity produced by stimulus trains (40 pulse, 4 Hz, 2 T) applied to S4 dorsal roots, before (control; top), after bathing the sacrocaudal segments in low-calcium/high-magnesium Kreb's saline (in a sacrocaudal (SC) chamber; middle), and after repeating the stimulus train following ejection of calcium from a micropipette onto the S3 segment (bottom). The region exposed to Ca^{2+} ejection (red) was controlled using a suction pipette positioned across the ejection site. The experimental bath was divided into sacrocaudal and thoracolumbar chambers using an L6/S1 Vaseline wall. (a) From Etlin A and Lev-Tov A, unpublished data. (b) Adapted from Strauss I and Lev-Tov A (2003) Neural pathways between sacrocaudal afferents and lumbar pattern generators in neonatal rats. *Journal of Neurophysiology* 89: 773–784.

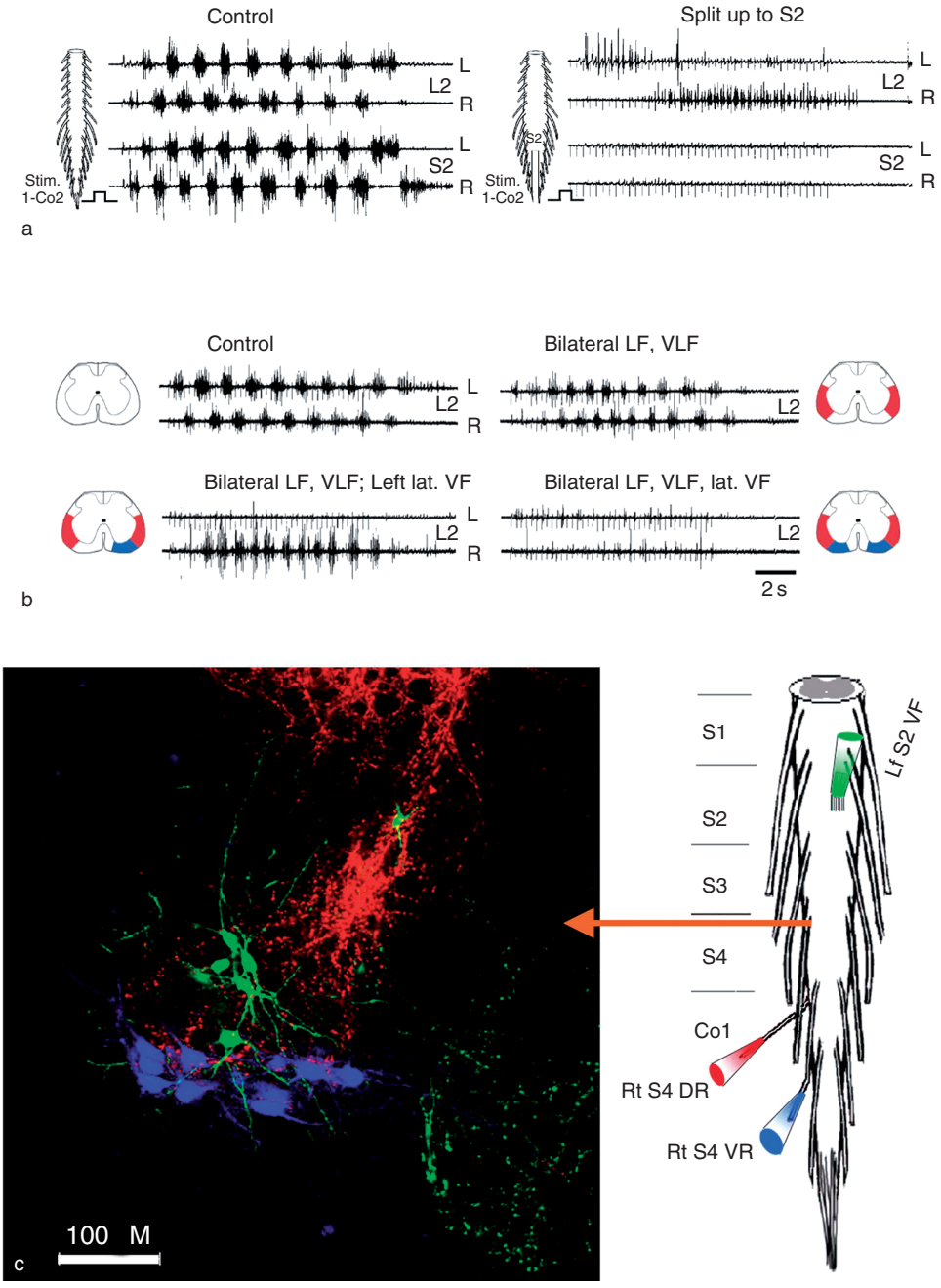

Figure 3 Sensory activation of locomotor CPGs by SCAs: the mediating pathways. (a, b) Sacrocaudal afferent stimulation activates sacrocaudal interneurons, the axons of which cross the sacrocaudal spinal cord and project rostrally through the ventral funiculi. The activity produced by stimulation of the left coccygeal Co2 dorsal root (40 pulse, 4 Hz, 1.5 T) and recorded from the left and right lumbar L2 and sacrocaudal S2 ventral roots (a; control) is severely impaired following a midsagittal split extending from the caudalmost end of the cord to caudal S2 (a; split to S2). The rhythmic activity induced in the lumbar cord by sacrocaudal afferent stimulation (40-pulse trains at 4 Hz and 1.5 T) (b; control) is virtually unaffected by bilateral lesion of the lateral (LF) and ventrolateral (VLF) funiculi at the L6–S1 junction (b; bilateral LF, VLF). Extension of the lesion to the lateral part of the left ventral funiculus (VF) blocks the activity at the ipsilateral side (b; bilateral LF, VLF; left lat. VF). Extension of the lesion to the right lateral VF (b; bilateral LF, VLF, lat. VF) virtually eliminates the rhythmic activity in the lumbar cord. (c) Sacrocaudal relay neurons and their afferent innervation. Projected confocal microscope image of a cross section at the rostral S4 segment in the neonatal mouse spinal cord. The right S4 dorsal roots (DR) were filled with Texas Red Dextran (red), the right S4 ventral roots (VR) were filled with Cascade Blue Dextran (blue), and a strip of the left ventral funiculus was filled with Fluorescein Dextran (green) at the rostral half of S2. The micrograph shows a dense ipsilateral neuropil of labeled ventral funiculus fibers with retrogradely labeled interneurons in the contralateral side of the segment. Putative contacts between the labeled interneurons and S4 afferents were found following 3-D reconstruction of the confocal images (not shown). (a, b) Adapted from Strauss I and Lev-Tov A (2003) Neural pathways between sacrocaudal afferents and lumbar pattern generators in neonatal rats. *Journal of Neurophysiology* 89: 773–784. (c) From Blivis D, Mentis GZ, O'Donovan MJ, and Lev-Tov A, unpublished data.

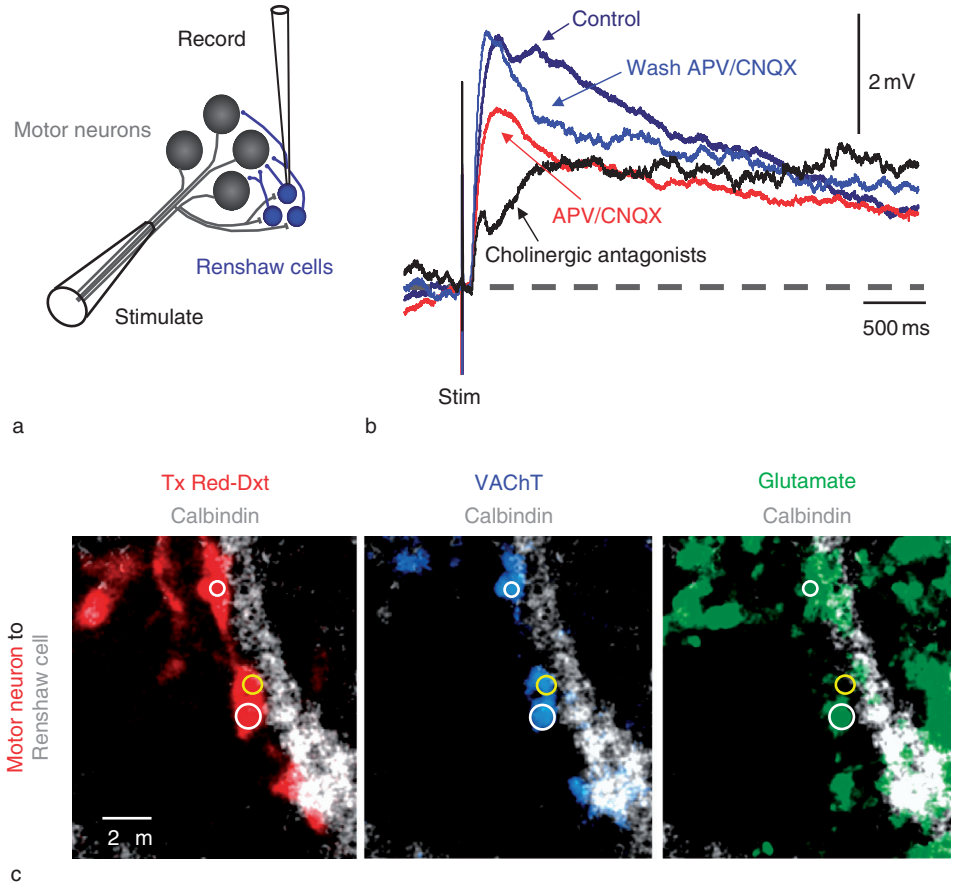

Figure 4 Synaptic potentials in a neonatal mouse Renshaw cell evoked by stimulation of motor axons are mediated by cholinergic NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate receptors. (a) Recording and stimulating arrangement. (b) Superimposed synaptic potentials recorded in a Renshaw cell in response to a single stimulus applied to motor axons of the sciatic nerve (control). The membrane potential of the cell was held at −70 mV throughout the recordings. Application of the glutamate receptor antagonists 2-amino-5-phosphonovaleric acid (APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) for 25 min depressed the amplitude of the response (APV/CNQX). The response fully recovered after 22 min of washout (wash APV/CNQX). Subsequent exposure to cholinergic antagonists depressed the amplitude of the potential. Each response is an average of five stimuli delivered at 0.1 Hz. (c) Glutamate is enriched in some motor neuron synaptic boutons contacting Renshaw cells. Confocal images of a calbindin-immunoreactive dendrite (gray) from a presumed Renshaw cell and several retrogradely labeled motor neuron varicosities labeled with Texas Red Dextran (TxRed-Dxt) and co-immunolabeled for the vesicular acetylcholine transporter (VAChT; blue) and glutamate (green). The white circles identify boutons immunoreactive for all three markers, and the yellow circles show a cholinergic motor neuron terminal without glutamate immunoreactivity. Adapted from Mentis GZ, Alvarez FJ, Bonnot A, et al. (2005) Noncholinergic excitatory actions of motoneurons in the neonatal mammalian spinal cord. *Proceedings of the National Academy of Sciences of the United States of America* 102: 7344–7349.

neurons and also from primary muscle afferents. Such cells exhibit many of the characteristics of adult Renshaw cells. These include their ventromedial location, their rapid firing following a single antidromic stimulus, and their expression of calbindin, a calcium-binding protein (see **Figure 4**). The number of terminals expressing a vesicular glutamate transporter specific for primary muscle afferents (VGLUT1) increases during the first three neonatal weeks and then decreases as the animal matures into adulthood. However, even in the adult mouse, some primary afferent terminals remain on putative Renshaw cells, although it has not yet been established if such terminals are physiologically active. In the neonatal mouse, the 1a inhibitory interneurons are believed to be located dorsally to the Renshaw cells but they have not yet been studied electrophysiologically. The great similarity between the synaptic connections of Renshaw cells and 1a inhibitory interneurons is perhaps not surprising given that they both derive from a common precursor population – the V1 class – of interneurons that express the *engrailed-1* transcription factor.

Collectively, findings in the adult cat and the neonatal mouse raise two possibilities: first, that the

properties of the Renshaw cell and the 1a inhibitory interneuron differ between the mouse and cat, and second, that the two interneuronal types differentiate from a common population that shares the connections of both cell classes. According to the second idea, Renshaw cells would differentiate from this precursor population by shedding their primary afferent connections whereas the future 1a inhibitory interneurons would selectively lose their motor neuronal inputs. It is also possible that these two identified interneuronal populations are not truly distinct but rather are drawn from a population continuum. At the extremes would be the classical Renshaw cell and 1a inhibitory interneuron as defined from studies of the adult cat. Between these extremes, might be a population that shares the connectivity patterns of both.

In the adult cat, the motor neuronal synaptic inputs to Renshaw cells were believed to be mediated entirely by the neurotransmitter acetylcholine. In the neonatal mouse, however, it has been found that the synaptic potentials produced in putative Renshaw cells by stimulation of motor neuron axons are mediated by cholinergic and glutamatergic/aspartergic neurotransmission (Figure 4). Such dual transmission has been observed in several different cell types in the developing nervous system, and its discovery has challenged the long-held view that motor neuron actions are exclusively cholinergic. At present, the function of this dual transmission is unknown. Clues to its possible role come from the observation that stimulation of the ventral roots in the neonatal mouse can trigger locomotor-like activity that persists in the presence of cholinergic antagonists but which is blocked by glutamatergic antagonists (Figure 5). This observation raises the possibility that neonatal motor neurons may make glutamatergic/aspartergic synaptic contacts with interneurons that have access to the locomotor central pattern generator.

Interneurons Involved in the Patterning of Motor Neuron Activity during Locomotion

In the past 10 years, several classes of developing spinal neurons have been identified based on their expression of transcription factors. As discussed in the previous section, it is not known how these developing neurons mature into the interneurons of the adult cord. Nonetheless, the participation of these primitive interneuronal types in the locomotor-like activity expressed by the neonatal cord has been studied by several groups. In considering this work, it is important to distinguish between two distinct aspects of locomotor behavior. The first is the rhythmicity exhibited by both motor neurons and interneurons, about which very little is known. The second is the timing of activity in the various motor neuron pools that innervate the limbs, the axial muscles, and the tail. For example, during locomotor-like activity both antagonistic and contralateral motor neurons alternate their discharge during each cycle whereas synergistic motor neurons fire bursts at the same time. Most studies of developing interneurons have focused on their contribution to the timing of motor neuron discharge.

One of the first interneuronal classes to be identified genetically was a group of ventrally located neurons expressing the ephrin-A4 (EphA4) receptor. This receptor and its ligand ephrin-B3 are thought to prevent axons from crossing the midline. Animals with a mutation in the EphA4 receptor exhibit an abnormal gait in which both hind limbs are activated in synchrony (hopping) rather than in alternation. The spinal neurons expressing the EphA4 are thought to be excitatory glutamatergic neurons, the contralateral projections of which are abnormally profuse in the mutant animals. More recently, it has been shown that cells expressing the EphA4 receptor form a heterogeneous population, although some of them fire rhythmically during locomotion and have direct glutamatergic projections to ipsilateral motor neurons. A second class of excitatory interneuron has been identified; it located in the lower thoracic and upper lumbar segments and expresses the homeobox gene HB9 transcription factor. This factor was known to be involved in consolidating motor neuron fate, so it was surprising to find it expressed in interneurons. Nonetheless, the $HB9^{+}$ interneurons have been shown to fire in phase with ipsilateral flexor motor neurons during locomotor-like activity. Dual recordings from these cells have shown that they are connected by electrical synapses that contribute to their synchronization during a flexor burst.

It is important to emphasize that experiments of this type do not reveal the functional role of the interneurons during locomotion. One way to approach this problem is to study locomotor deficits when particular interneurons have been prevented from developing using genetic means. This has been accomplished in mice by knocking out the homeobox DBX1 transcription factor, which results in the loss of the V0 class of interneuronal precursors. These are inhibitory interneurons that send their axons across the midline of the spinal cord. Animals lacking V0 interneurons have defects of left/right alternation suggesting that some of the V0 interneurons are involved in regulating the timing of contralateral motor neuron activity during locomotion. The same approach has been used to study a second class of inhibitory interneurons with projections that are

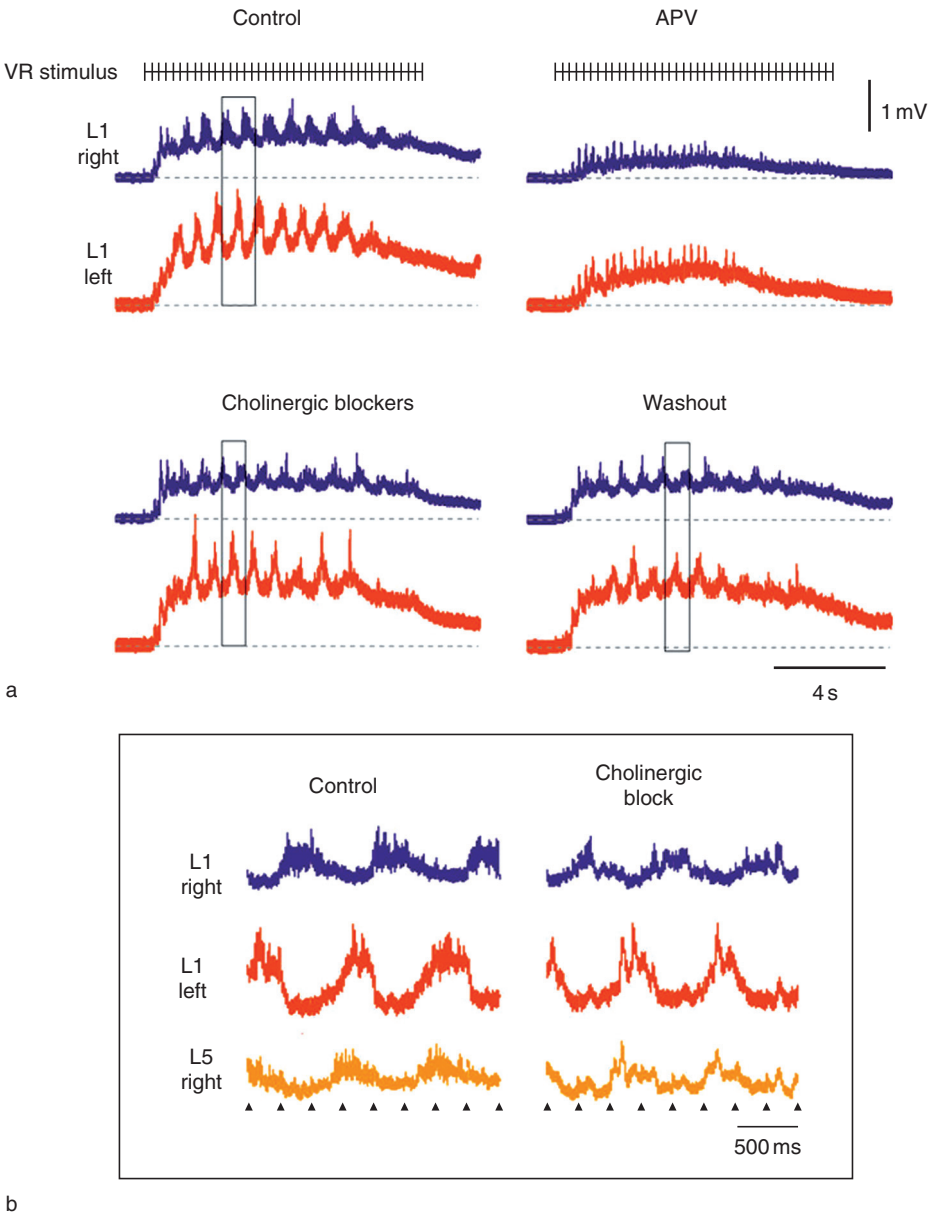

Figure 5 Locomotor-like activity in the neonatal mouse (P3) can be triggered by motor axon stimulation. (a) Ventral root recordings (L1 right and L1 left) during an episode locomotor-like activity induced by a train of stimuli applied to the motor axons of the sciatic nerve (dorsal roots cut). The recordings were made before (control), after 1 h of cholinergic block (mecamylamine 50μm, DHβE 50μm, atropine 5μm) followed by the NMDA receptor antagonist APV (100 μm) and washout of all drugs. The stimuli are shown at the top of the traces. Note that the alternating locomotor-like rhythm persists in the presence of cholinergic antagonists (see expanded traces in (b)) but is abolished by APV. (b) Expanded timescale display of the traces shown in (a), and of the simultaneously recorded L5 activity (not shown in (a)). Adapted from Mentis GZ, Alvarez FJ, Bonnot A, et al. (2005) Noncholinergic excitatory actions of motoneurons in the neonatal mammalian spinal cord. *Proceedings of the National Academy of Sciences of the United States of America* 102: 7344–7349.

believed to be exclusively ipsilateral. Knockout of this class (V1) of interneuron results in a slowing of the locomotor rhythm induced by drugs.

Intracellular recordings from contralaterally projecting commissural interneurons located in the ventromedial part of the lumbar cord have revealed a heterogeneous set of firing patterns and connectivity. Included in this population are cells projecting monosynaptic excitation or inhibition to contralateral motor neurons and other cells with connectivity that changes from rest to locomotor activity. It is likely that these recordings included some of the cells derived from the V0 category that have been implicated in the control of left/right alternation.

See also: Central Pattern Generators; Central Pattern Generators: Sensory Feedback; Motor Primitives; Pattern Generation; Primate Interneurons; Sensorimotor Plasticity and Control of Movement Following Spinal Cord Injury + D18; Spinal Motor Neurons: Properties.

Further Reading

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