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Genetic analysis of left-right coordination of locomotion

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1. ABSTRACT

While there is a rather large amount of data from pharmacological and anatomical studies of the murine locomotor CPG network, comprehensive information regarding the cellular and functional properties of the neuronal populations is lacking. Here, we describe concepts arising from genetic studies of the locomotor network with a focus on commissural interneurons regulating left-right coordination. In particular, this involves several families of axon guidance molecules relevant for midline crossing. We also describe recent advances within the field of neural circuit analysis, including imaging, genetic inactivation and optogenetic strategies, which are applicable to locomotor circuits. Such efforts, for example by using available genetic markers, should substantially increase our possibilities to decipher the functionality of spinal cord neuronal networks.

2. INTRODUCTION

Bilateral coordination is required when both sides of the body are involved in movements. Variants of bilateral coordination include: 1) simultaneous movements such as when using a rolling pin, 2) alternating movements when walking or running and 3) non-repetitive synchronized movements for example when you stabilize a paper with one hand while writing with the other. Such tasks are required for everyday life and needed in animals of any complexity. Dorso-ventral undulating movements in C. elegans, six legged walking in Drosophila and swimming in zebrafish adult and larvae are all examples of bilateral coordination essential for movement towards e.g. food and mating. While such coordination may appear simple at first glance, the ever-changing environment requires rapid adaptation of movements, which demands swift and efficient control by the involved neuronal

networks. The number of neuronal populations participating to reach a sufficient degree of control is currently unknown, however, as we shall see, genetic methods now allow for a progressively finer map of identified subpopulations and their possible participation in bilateral locomotor coordination. While the animals exemplified above are genetically tractable (1), in this review we focus on genetic experiments in mouse aimed at deciphering left-right coordination.

The flow of communication between the two sides of the body requires commissural interneurons (CINs), which are defined by their projections to the contralateral side of the body. Spinal CINs interneurons, which have local axons that cross over to the other side of the spinal cord from where their cell bodies are located, form essential elements of locomotor networks. In mammals, many CINs are rhythmically active during locomotor-like activity and have been considered to be important parts of the central pattern generator (CPG) network. Locomotor CPGs in the spinal cord are neuronal networks that produce rhythmic activities necessary for coordinated limb movements (2-5). The CPGs are mainly responsible for the generation of a stable rhythm while it also needs coordination of flexors and extensors as well as over the midline. Surgical transection of the ventral commissure, which cuts all contralateral projections, results in a disruption of left-right alternating motor activity (6). Both excitatory and inhibitory CINs are considered to coordinate left-right activities during locomotion (7-10) and a working model suggests two separate circuitries responsible for left-right alternation and synchrony (11). However, the precise identities of cells regulating left-right alternation and/or synchrony remain unclear.

3. LOCOMOTOR COORDINATION

3.1. Establishment of coordinated locomotor activity

Rhythmic spontaneous activity from a small circuit in the isolated mouse spinal cord composed of motor neurons and excitatory GABAergic interneurons can be detected already at embryonic day (E)11 (12). This activity is a prerequisite for the correct execution of early axon pathfinding decisions and wiring (13, 14). Subsequent occasional bursts of spontaneous activity can be detected until E18 and are likely to be signs of network maturation (15). Coupled bilateral rhythmic bursting patterns in embryonic mouse preparations can be induced by 5-HT already from E12 (16) and lumbar bursts across the cord at this stage are relatively synchronous. At E15, left-right activities become progressively alternate and strictly alternating patterns are seen from E18 and maintained after birth. In accordance, recordings of coordinated rhythmic activity from *in vitro* preparations have revealed two interesting changes during development of CPG coordination. First, the rhythmic activity is synchronous between E14.5 and E16.5 in the rat, both between the two sides of the spinal cord and between flexion and extension. At E18.5 in the rat, the synchrony between the two sides of the spinal cord has switched to alternation, but the activity from flexion and extension outputs remain synchronous. And finally, at E20.5, CPG coordination has reached its mature state when also the synchronous flexion and extension activity has switched to alternation (17). For the mouse, the period of general synchronous activity take place between E12 and E14 and the switch to left-right alternation has manifested itself at E18 (16). Early synchrony between left and right is likely mediated by commissural excitatory fibers connecting the rhythm-generating networks on each side of the spinal cord (18). Interestingly, these early excitatory connections are mediated via GABAA receptors and when the synchronous activity later switches to left-right alternation, the GABAA receptors instead mediate inhibition. This switch in signaling response during development coincides with the onset of the neuron-specific potassium chloride co-transporter 2 (KCC2) expression. Thus, the maturation of left-right coordinated activity during development likely depends on the timing of postsynaptic KCC2 co-transporter expression (19). There is an additional switch in transmitter phenotype when the glycine receptor takes over the inhibitory role from the GABAA receptor but the functional consequence of this switch is not clear (20). Considering that a functionally mature locomotor CPG can also be defined as a network able to produce a rhythm in animals carrying their own weight, maturation of the CPG in mice completes around postnatal day (P)11. While each of NMDA, 5-HT or dopamine can initiate and produce rhythmic activity during early stages (E14 – P7, (21-23)), a combined application of all three neurochemicals is required for the generation of rhythmic alternation at P12 (24). These results suggest that the basic neuronal CPG circuitry for bilateral coordination is established around E14.5 or earlier and that the following maturation is dependent on neurotransmitter and receptor phenotypes rather than rewiring of the underlying interneuron network.

3.1.1. Location and properties of left-right components

The main neuronal components underlying left-right locomotor coordination are CINs whose axons cross the midline via the ventral commissure. Lesion experiments in neonatal spinal cords have shown that the dorsal spinal cord is dispensable for rhythmic and coordinated locomotor activity (6) and that normal left-right alternating locomotion disappears after cutting the ventral commissure. These experiments have demonstrated that basic left-right coordination is mediated by ventrally located CINs, at least in the perinatal rat spinal cord.

Rodent CINs can be divided into subpopulations based on their pattern of projections; more than two segments projecting intersegmental CINs and locally projecting intrasegmental CINs. Intersegmental CINs can be further subdivided into ascending (aCINs), descending (dCIN) and bifurcating (adCINs) CINs (25-28). In addition to being involved in hindlimb coordination, aCINs with long ascending fibers provide rhythmic signals to the forelimb CPG region of the spinal cord and part of this drive is crossed inhibition (29). dCINs serve a role in providing direct input to motor neuron activity in more caudal spinal segments (30, 31) but also give input to interneurons on the contralateral side (32, 33). Intrasegmental connections are likely to play a direct role in organizing the left-right coordination between

segmental, homonymous muscles. Similar to other vertebrates such as the lamprey, the functional murine CPG network has been suggested to include alternating glutamatergic excitation and glycinergic inhibition (31, 34, 35). Both excitatory CINs using glutamate as a neurotransmitter and inhibitory populations of CINs using glycine or GABA have been identified (31, 34, 35) and more recent studies in rat and mouse spinal cord have shown that excitatory CIN and terminals are less abundant than inhibitory ones (36-38). Pharmacological studies in which glycine receptors were blocked have indicated that inhibitory neurons play an important role in the alternation between left and right limbs as well as alternation in the activation of ipsilateral flexor and extensor muscles (39). Also neuromodulatory connections are likely to coordinate activities over the midline. Recent work assessing 5-HT7 receptor knockout mice during fictive and adult locomotion has shown the importance of serotonergic activation of 5-HT7 receptors for left-right alternation activities (40). A working model for left-right coordination suggests two parallel systems of CINs involved in CPG function (Figure.1a). 1) A left-right alternation circuitry that consists of inhibitory CINs and provides fast monosynaptic inhibition onto contralateral motor neurons (MNs) (39) and a set of excitatory CINs that indirectly inhibit MNs by activation of contralateral inhibitory interneurons (41). This system is believed to be necessary for contralateral silencing during normal left-right alternating locomotion. However, a complete deletion of vGluT2, the most abundant vesicular glutamate transporter in the spinal cord, strongly affected respiratory circuitry while leaving locomotor rhythm and left-right coordination intact, suggesting that glutamate release mediated by vGluT2 is non-essential for left-right coordination (42, 43). 2) A left-right synchrony circuitry, which consists of a set of excitatory CINs directly activating contralateral MNs (11). The latter has been proposed to be involved in binding left-right segmental activity (e.g. during hopping) most likely by overriding the otherwise dominant alternation circuitry. In addition, application of the glycine receptor inhibitor, strychnine, to mouse spinal cords with uncoordinated (E15) or alternating (E18 and older) activity has been shown to transform the activity to synchrony (16). This suggests that inhibitory signaling is important for normal left-right alternation, and identifies the presence of a bilateral synchronous bursting activity independent of glycinergic signaling, which, under normal conditions, is suppressed.

3.1.2. Targeting of genetically defined subpopulations in functional studies of locomotor coordination

In the spinal cord, clear pools are formed by the motor neurons; however, the interneurons are more difficult to discriminate. Therefore, studies aimed to identify the subcomponents of the locomotor CPG, as well as ancillary components responsible for left-right coordination, are challenging but nevertheless absolutely critical to dissect the development of locomotor neuronal circuitry, and to understand neuronal circuit operating principles in general. Subpopulations of spinal cord interneurons can be identified during development by their expression of specific homeodomain transcription factors (2, 44, 45). Dorsal progenitor cells give rise to six early classes of neurons, dI1 to dI6, and ventral progenitors give rise to motor neurons and four classes of interneurons, V0 to V3. Several of these early classes of neurons have been found to give rise to CINs (36, 46-50). Out of the ventral subtypes, the V0 and V3 populations are considered to give rise to CINs and have been investigated in genetic studies on left-right locomotor coordination.

V0 interneurons arise from p0 progenitor cells expressing the Dbx1 homeodomain (HD) protein and consist of two populations of cells, one of which express the HD protein Evx1 (46, 49, 50). When leaving the proliferative zone, these cells take on a ventral migratory route to settle in lamina VII /VIII and extend their axons rostral on the contralateral side of the spinal cord. Dbx1 is expressed in progenitor cells giving rise to both V0V and V0D neurons, whereas Evx1 is expressed in postmitotic V0V interneurons only. Deletion of Evx1 resulted in a selective loss of V0V interneurons, whereas a deletion of Dbx1 led to a loss of both V0V and V0D. While a loss of V0V neurons in Evx1 null mice gave a normal pattern of coordination during *in vitro* locomotion, the loss of both V0V and V0D in Dbx1 knockout mice led to intermitted episodes of synchrony between left and right ventral roots. The V0 population consists of 70% inhibitory and 30% excitatory interneurons (48). Evx1+ V0V neurons express lower or no levels of Pax2 protein while V0D neurons express high levels of Pax2 (36). Pax2 has been shown to mark an inhibitory cell fate in dorsal neurons (51), which if this applies also to the V0 population, would suggest that V0D neurons are inhibitory, while V0V neurons would constitute the majority of the excitatory V0 neurons. Taken together, an additive effect of the loss of both V0 populations could explain the observed phenotype. Alternatively, a specific loss of the inhibitory V0D population as part of the dominant left-right circuitry for bilateral CPG coordination could result in a partial strengthening of the synchrony circuit with a functional consequence of synchronous episodes of locomotion (Figure 1b).

Sim1-expressing V3 neurons arise from Nkx2.2-positive p3 progenitors and are predominantly excitatory. These cells appear to settle close to their origin in lamina VIII (52) and might be heterogeneous in regard to axonal projections, with both but mostly commissural and some ipsilateral fibers (27, 53). Use of a Cre/TeNT strategy resulting in impeded synaptic transmission from Sim1-positive neurons has shown that the V3 population establishes a regular and balanced motor rhythm distributing excitatory drive over the midline (53)(Figure 1e).

Although ipsilateral populations of neurons do not directly regulate binding of bilateral coordination, they might provide ipsilateral input to CINs and vice versa, several CINs have been shown to synapse on ipsilateral neurons on the opposite site of the spinal cord (11, 54). For this reason, genetic studies of specific ipsilateral subpopulations with regard to their function during locomotion may be valuable to also understand left-right activities. P1 progenitors expressing HD proteins, Dbx2

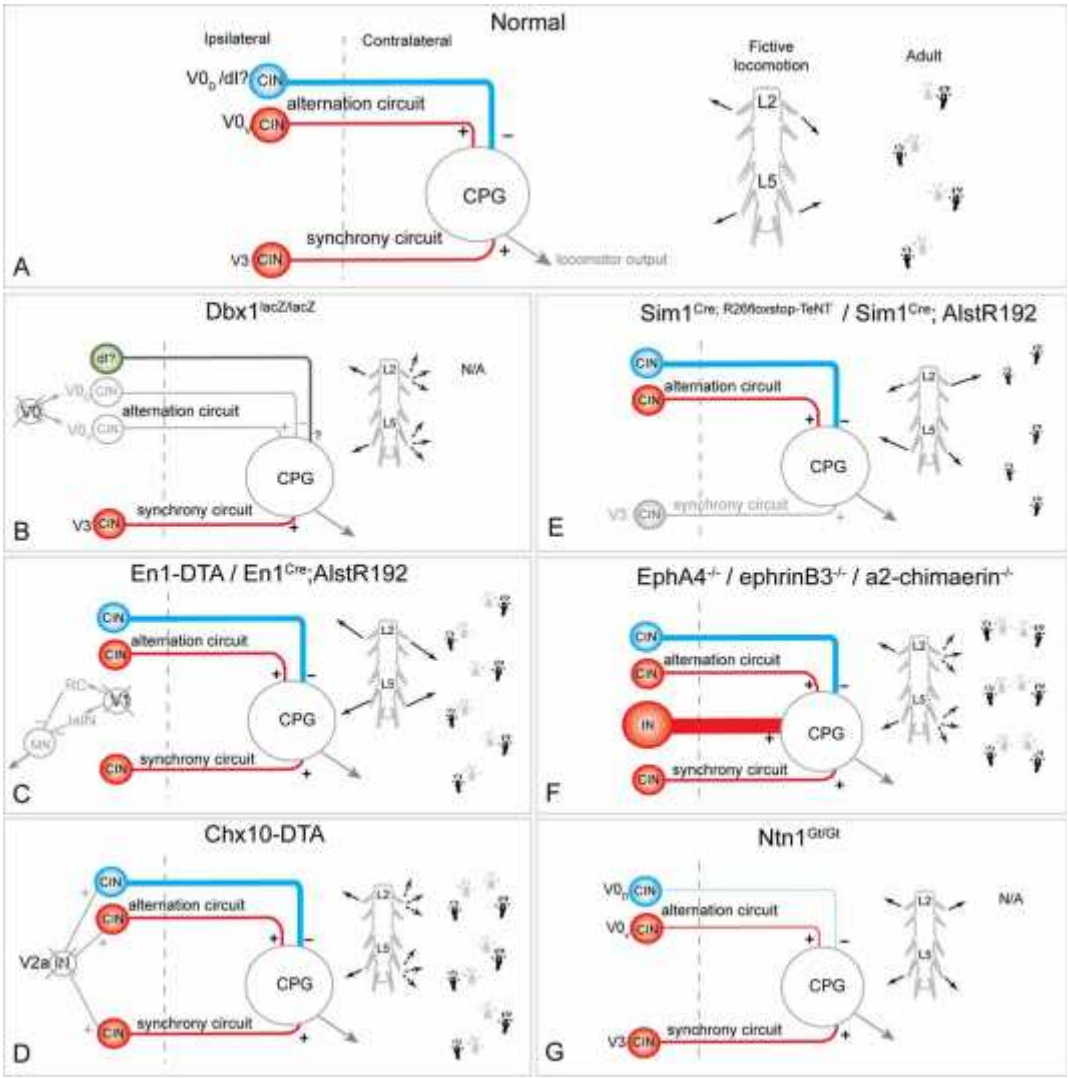

Figure 1. CINs related left–right coordinating circuits in the rodent CPG based on electrophysiology and genetic ablation/silencing experiments. (A) During normal locomotion two separate circuitries are responsible for left-right coordination. A left–right alternation circuitry acting via inhibitory CINs (blue), or via excitatory CINs (red) on the contralateral CPG and a excitatory synchrony circuit (red) acting directly on motor neurons (11). The dominant locomotor output from the combined circuitry is alternation both in perinatal fictive locomotion and the adult animal. (B) The ablation of Dbx1 positive V0 progenitor cells generating glutamatergic V0V and GABAergic/Glycinergic V0D CINs in Dbx1lacZ/lacZ mice leads to frequent episodes of synchrony in fictive locomotion (illustrated by the dashed arrows), adult locomotion is not applicable as the mutants die at birth (48). (C) Deletion of V1 neurons in En-1 DTA mice and acute silencing of V1 neurons via the allatostatin receptor slow the speed of locomotion but normal left-right coordination activities remain (62). (D) The deletion of V2a neurons in Chx10-DTA mice contributing to the locomotor CPG network via contralateral CIN results in uncoordinated fictive and adult locomotion (54). (E) Both selective neurotransmission block of V3 neurons in Sim1Cre; R26floxstop-TeNT and acute silencing of Sim1+ V3 neurons in Sim1Cre; AlstR192 showed increased variability in the length of the step cycle period coupled with an asymmetry in the duration of flexor bursts between the left and right halves of the spinal cord (53). (F) In EphA4, ephrinB3 and a2-chimaerin mutant mice, the ipsilateral projecting interneurons aberrantly cross to the contralateral side, resulting in a locomotor output drifting between alternation and synchrony between the right and left ventral roots and the rabbit-like gait seen in adult mice (87). (G) Albeit a striking loss of CINs in Ntn1 mutant mice the synchrony circuitry remains intact and the alternating left- right coordination switches to strict synchrony (36). Vertical dotted lines indicate the midline. Schematics of spinal cords illustrate ventral root activity with arrows pointing up and down represent activity and inactivity, respectively. Black paws represent hind limbs and grey front limbs.

and Nkx6.2, give rise to Engrailed 1 (En1)-positive V1 interneurons (55). These cells migrate ventrolateral to reach their position in lamina VII and have been found to develop local projections (56). Studies of En1/2-expressing V1

interneurons have shown that in "simpler" vertebrates such as the fish (57) and frog (58), these neurons represent a homogenous cell population of ipsilateral glycinergic inhibitory interneurons that play important roles in motor control and sensory gating during swimming. Interestingly, although En1/2-expressing V1 interneurons share common features among species, such as transmitter phenotype and axonal projections, they appear to have more heterogeneous functions in higher vertebrates (59). A subset of V1 interneurons seems to generate many of the different local circuit inhibitory interneurons that are present in the mammalian spinal cord. These include the Renshaw cells, which mediate recurrent inhibition to motor neurons, as well as the Ia inhibitory interneurons that receive input from Ia sensory afferent neurons of the spinal reflex pathway (60, 61). Such functional diversification of an early embryonic class of neurons in higher vertebrates might represent a common rationale for generating circuits controlling more complex locomotor movements. However, studies of fictive locomotion in neonatal preparations of En1 null 'knock-in' mice and acute silencing of V1 neurons via the allatostatin receptor have shown to slow the speed of locomotion but with remaining normal left-right coordination activities (62) (Figure 1c).

V2 interneurons develop from Irx3 and Nkx6.1-expressing p2 progenitors into two separate populations of postmitotic cells, one expressing Chx10 and Lhx3 and the other expressing Gata2 and Gata3 (63, 64). The V2 interneurons migrate laterally to their location in lamina VII. Ablation of V2a interneurons with a Chx10-DTA strategy results in greater variability in cycle period and amplitude of locomotor bursts (54) during fictive locomotion and adult mice show a speed dependent loss of left-right alternation defined by a transition to synchronous gait at high speed (65) (Figure.1d).

Thus, ventral-originating populations of neurons are involved in various aspects of locomotion. With regard to left-right coordination, the rhythms of fictive locomotion were irregular with episodes of synchrony and alternation (V0; (48)) or drifting in and out of strict alternation (V2a; (54)), together perhaps best described as uncoordinated phenotypes. Consequently, it seems likely that multiple neuronal subtypes originating from several ventral progenitor domains are involved in the different aspects of left-right coordination. In addition, while genetic studies have so far focused on the role of ventral interneurons in the CPG network, it has been reported that during development dorsally born neurons migrate ventrally (46-48, 50), suggesting the possibility that neurons originating from the dorsal spinal cord might participate in ventral located circuitries. Indeed, dorsal originating interneuron populations also extend commissural projections (36, 66-68), and are therefore candidates regulating midline coordination during locomotion. Of the dorsal CIN populations, inhibitory dI6 neurons, which settle in lamina VII and VIII are the most promising candidate neurons for left-right alternating circuitry. This is supported by the idea that inhibitory commissural connections are the major pathways responsible for coordinating the left-right phasing during locomotion (39, 48, 69-72). A possible role of presumable excitatory dI5 or dI3 neurons for CPG coordination remains to be determined. Interestingly, dI3 neurons were recently demonstrated to directly contact MNs by rabies virus tracing (73) and dI4 neurons form contacts on Ia afferent terminals near MNs (74). Finally, dI1-dI2 interneurons are not likely to contribute to CPG coordination, since they have been suggested as part of ascending pathways including the spinocerebellar and the spinothalamic tract (46, 47). In any case, we would like to stress the importance of extending future studies to investigate neurons and their role for bilateral coordination during locomotion to neurons derived also from the dorsal neuroepithelium.

3.2. Midline axon guidance and bilateral coordination

During vertebrate development, the floor plate and ventral midline play a critical role in patterning, neuronal fates and projections within the spinal cord. Diffusible proteins, morphogens and guidance molecules, specify neuronal cell fates along the dorsoventral axis and represent guidance cues for migrating neurons, growing axons, and dendrites (75, 76). Morphogens such as bone morphogenic proteins (BMPs), Shh, and Wnts have been shown to primarily pattern neuronal fate but also to guide migrating neurons within the spinal cord (45, 77). The subsequent formation of functional neuronal circuitry during development is dependent on the correct guidance of axons to their targets. The four families of known axon guidance cues and their receptors have grown considerably the last decade including the ephrins with Eph receptors, Slits with their Robo receptors, semaphorins with neuropilin and plexin co-receptors, and netrins with DCC, Unc-5/RCM and neogenin receptors (76, 78, 79). These guidance molecules can be divided in secreted attractants and repellents, or membrane attached attractants and repellents (80, 81). Netrins, Slits and some semaphorins are secreted molecules associated with cells or extracellular matrix and can act as attractants or repellents in different contexts (82-84). Ephrins and semaphorins are expressed at the cell surface and act primarily as repellents but have also been shown to promote attraction or adhesion (78, 85). Several mutant mice, deficient for axon guidance molecules show aberrant locomotor phenotypes regarding left-right coordination. Both CINs and ipsilateral neuron populations need axon guidance cues (56). Thus, the aberrant locomotor output observed in mutants affecting axon guidance could be due to misprojections of either ipsilateral axons or CIN, or both. However, a detailed examination of the mechanisms underlying the phenotype in these genetic models can help us understand and identify crucial components of functional CPG circuitry.

3.2.1. Eph/ephrins

Many axon guidance processes during development are regulated by the Eph/ephrin system. In the embryonic spinal cord, early subsets of motor neurons as well as scattered ipsilateral interneurons have been shown to express EphA4. The EphA4 ligand ephrinB3, on the other hand, is expressed in the floor plate and as the spinal midline is formed, also in the ventricular zone and roof plate (86) . Here, EphA4/ephrinB3 interaction typically results in growth cone retraction and collapse to prevent midline crossing. EphA4-null mice represent the first

example with a distinct loss of rhythmic alternation connected to local spinal CPG dysfunction (87) (Figure.1f). This phenotype has been explained by misguidance of excitatory spinal cord local interneurons over the midline, which would normally project to ipsilateral targets. Mice with a deletion of ephrinB3 mimic this spinal phenotype, demonstrating the role of EphA4/ephrinB3 signaling in the development of the neuronal network that controls bilateral walking (87). However, the number of aberrant fibers crossing in these mice is extensive and it is unlikely that all of the affected neurons are functional components of the CPG. Nevertheless, a distinct population of EphA4-positive neurons responsible for the deviant locomotor coordination has so far not been defined.

Ephrin/Eph receptor interaction can lead to a complex bidirectional forward and reverse signaling both through the receptor and the membrane bound ligand. Mice that have impaired kinase activity of EphA4 (ephA4KD/KD and ephA42F/2F) but not mice expressing a truncated form of ephrinB3 show a similar abnormal locomotor phenotype as ephrinB3 and ephA4 null mice (88, 89). Thus forward but not reverse signaling seems to be essential for the formation of the underlying motor circuitry. Further studies suggest a role of α2-chimaerin as a downstream molecule of EphA4 signaling. Here, genetic ablation of α2-chimaerin results in either uncoordinated or synchronous left-right activities in neonatal mice (90, 91) (Figure 1f).

3.2.2. Netrin-DCC

Netrin-1 has an important role for axon pathfinding and neuronal migration during the development of the CNS. Among others it has been shown to act as a diffusible floorplate chemotropic cue for commissural axons in the spinal cord (92, 93). Netrin-1 represents the first example of an axon guidance molecule that when dysfunctional, completely switches the alternating left-right coordination to strict synchrony in fictive locomotion (36) (Figure 1g). This phenotype can be explained by the selective loss of CINs from distinct developmental progenitor domains. In Netrin-1 mutant mice, only axons from the most ventral originating excitatory V3 neurons cross correctly, which results in a significant reduced number of CINs and a changed neurotransmitter balance over the midline. This suggests that the V3 population is an important component of the left-right synchrony circuitry. Since the phenotype of Netrin-1 mutant mice is different from Dbx1 mutants in that it is consistently left-right synchronous, also the V0V population, in addition to the V3 population, could be part of the left-right synchrony circuit while V0D and additional dorsally originating CIN subpopulations could be vital for normal left-right alternation. The study of Netrin-deficient mice not only showed its importance for the proper development of the neuronal circuit underlying normal left-right alternation, but also helped to identify developmental subpopulations of CINs implicated in different aspects of bilateral coordination. DCC promotes commissural axon outgrowth *in vivo* through binding to netrin-1 (94-96). Similar anatomical phenotypes produced by deletions of Dcc and Netrin-1 in mice further support Netrin-DCC as a functional ligand-receptor pair during midline guidance in the spinal cord (97, 98). However the role of DCC mediated axon guidance for CPG functionality is yet to be resolved.

3.2.3. Slit-robo

There are three mammalian Slit genes (Slit1–Slit3) expressed by midline cells and four Robo genes (Robo1-Robo4) expressed in CNS neurons (99-103). Robo1 and Robo2 protein expression is low as commissural growth cones extend toward and across the midline, but high after crossing (104). Robo3, which is expressed in CINs, occurs in two distinct splice variants with opposite functions (102, 105). The Robo3.1 isoform is expressed on pre-crossing commissural axons and suppresses premature responsiveness to Slits, thereby allowing midline crossing, whereas Robo3.2 is expressed on post-crossing axons thus preventing re-crossing (105). In mice, mutations in all three Slit genes lead to axons aberrantly crossing or stalling at the midline (104, 106, 107). Effects on motor and sensory motor behaviors have been reported in Robo3-deficient humans, zebrafish and mice (108-111). Mutations in human Robo3 were discovered in patients with a rare autosomal recessive disease named horizontal gaze palsy with progressive scoliosis (HGPPS). Characteristic for this syndrome is the aberrant ipsilateral projections of both the descending corticospinal tract motor projections and the ascending lemniscal sensory projections. Additionally HGPPS patients are unable to perform conjugate lateral eye movements (109, 112). In Robo3/twitch twice double-mutant zebrafish defects in eye movements and balance have been reported (110). In Robo3 knockout mice, commissural axons are prematurely responsive to midline Slit repellents resulting in ipsilateral rather than contralateral projections giving rise to motor and sensory deficit (102, 113, 114). However behavioral studies on Robo3-null mice are prevented since they die shortly after birth. To circumvent this drawback, a Robo3 conditional knockout line was generated providing a genetic tool for studying specific subsets of CINs by crossings with transgenic mice expressing Cre recombinase (111). The genetic manipulation of Robo3 in spinal cord could thus provide a promising avenue for characterizing specific CIN subpopulations in the left-right circuitry.

3.2.4. Semaphorins

Semaphorins are a large and diverse family of highly conserved cell surface and secreted guidance molecules (85). They are mediating both long- and short-range signals in guidance of cell migration, directional turning of developing axons, regulation of intra-axonal vesicular transport and synaptic transmission (115-119). Two main protein families have been identified as receptors for semaphorins, the transmembrane proteins plexins (Plexs) and the neuropilins (NPs) (120). Genetic analysis of semaphorin function in flies and in mice suggests that they primarily act as short-range inhibitory cues that deflect axons away from inappropriate regions, or guide them through repulsive corridors (121, 122). So far, only a limited number of semaphorin and neuropilin gene knockouts have been analyzed in the mouse. Neuropilin-2 mutant mice for example show severe guidance defects,

including axons stalling in the midline, overshooting to the contralateral side of the spinal cord and randomly projecting along the anterior-posterior axis as well as defects in the dorsal funiculus and anterior commissure of the brain (123, 124). A recent study showed that secreted semaphorin 3A (Sema3A) induces the identity of neurites of Xenopus spinal commissural interneurons (xSCINs) by activating CaV2.3 channels. Thus, these results suggest that Semaphorins not only have a guidance function, but possible other roles such as control of neurite identity during circuit formation and assembly (Nishiyama *et al.* 2011). Out of the here described set of axon guidance molecules, the semaphorins and their receptors are the least explored in the functional analysis of left-right coordination and future examination of these molecules may well be useful to further promote the understanding of bilateral circuit formation and function.

3.3. Challenges in genetic analyses of left-right coordination

Many studies take advantage of the possibility to analyze fictive locomotion in the in-vitro preparation of the spinal cord (125, 126). While certainly useful, it must be kept in mind that this preparation has some caveats. First, the signals from the ventral root L2 and L5 are an approximation of signals to extensor and flexor musculature; motor pools are elongated inside the spinal cord and the ventral roots do not exclusively contain either flexor or extensor signals (reviewed in (127)). Typically, motor pools span two to four spinal cord segments along the rostrocaudal axis. This is a minor problem if the conclusions drawn from an analysis of ventral root activities refrain from articulating distinct correlations between roots and function. Moreover, the *in vitro* preparation offers study of an incompletely matured network for locomotion. It is far from certain that the neuronal circuitries in the perinatal spinal cord resemble the circuitries responsible for adult walking. With regard to CINs and left-right coordination, the early bilateral communication observed during embryonic stages progressively develops and specifies CINs into populations with specialized tasks, including excitatory, inhibitory, intra- and intersegmental roles. Integration of the anatomical location, projection pattern and neurotransmitter phenotype of spinal CINs indicates a considerable diversity in the identity and connectivity of CINs (37). In addition, CINs in the spinal cord have a wide range of diverse sensory functions. Thus, development and specification of CINs must be considered likely to continue at least until the locomotor CPG has reached its final state. A couple of examples support this notion.

In EphA4-null mutant adult animals, the hindlimb locomotor pattern is exclusively synchronous during normal uninterrupted movement. Although such mice are able to move their hindlimbs in alternation, this most often happens when locomotion is interrupted, such as when presented with an obstacle or a turn (unpublished observations). In contrast, fictive locomotion analysis indicated that perinatal EphA4-mutant mice drift between alternation and synchrony, suggesting a stronger influence from aberrantly crossing fibers at more mature stages (87) (Figure 1f). It was demonstrated that these aberrant fibers might be local and originate from higher brain centers (88, 89). Another example is constituted by the Dcckanga mice, which show a mild to severe inability to maintain an upright position and frequently move their hindlimbs in synchrony (128). However during fictive locomotion in perinatal Dcckanga/kanga mice most preparations exhibited a clear alternating left-right activity, while only rarely abnormal coordination between left and right roots with bursts shifting to synchrony was observed (Rabe Bernhardt *et al.* unpublished data). Nevertheless, the perinatal network, regardless of its state of maturity, offers a network amenable for studies of principles of network function.

The specific targeting of midline axon guidance molecules to rewire circuitries *in vivo* has proven to be an efficient method for a better understanding of how left-right coordination circuitry develops and functions. However, this approach also has caveats. For example, when axons are misguided over the midline or prevented from crossing the midline, it is not straightforward to establish where the axons go instead. They might establish irregular connections with subsequent potential effects on left-right coordination, something that one ideally should control for. Similarly, when transcription factors are deleted or when the promoters of transcription factors are used to regulate the onset of Cre or any type of modifying protein, early effects must be expected that may lead to compensatory mechanisms when the actual experiments are carried out. This may impact adult locomotion analysis the most since the time between onset of genetic modification and phenotypic observation allows for more compensatory mechanisms. As we will describe in the following paragraphs continuous efforts, methods and reagents more suitable for analysis also of adult locomotion can help to resolve principles of the fully matured locomotor circuit.

3.3.1. Novel genetic techniques

Genetic approaches provide versatile tools to dissect and understand neuronal circuits not only at the molecular and cellular levels, but also at the network and physiological level. For this methodology to become efficient, it has been essential to identify the participating neuronal subpopulations in a given circuit. For example, the gene encoding the dopamine transporter (DAT) provides an excellent tool to genetically target dopaminergic neurons and the DAT promoter has successfully been used in several genetic experiments. It should be noted that use of genetic markers that identify different early classes of progenitor cells can be problematic since they are often down-regulated at later developmental stages, and since the progenitors often give rise to large and heterogeneous population of neurons. In a genetic investigation of the mid–late stages of mouse embryonic brain development, 11,061 genes were significantly expressed in at least one of the four different stages (E12, E15, E18 and P0) and 11.4% of these had a significant difference in their developmental expression (129). Thus, for efficient use of genetic approaches, it is critical to characterize the genetic markers well and in particular their pattern of expression in adults and during development. With an extensive collection of gene

expression patterns in our hands (GenePaint; www.genepaint.org and Allen Brain Atlas; www.brainatlas.org), the possibilities of using advanced mouse genetics to selectively interfere with distinct subpopulations of neurons are enormous. Neuronal circuits are now analyzed with precision at the cellular and molecular level using genetically engineered mice, which take advantage of the promoters regulating the expression pattern of the marker candidates to express versatile tools, including the Cre protein. The Cre-loxP system has become a standard approach for performing region-specific gene inactivation in mice (130) and is a system with high reliability *in vivo*. Suitable reporter mice are readily available that after Cre-mediated excision of a STOP cassette, express various proteins to visualize neurons, usually by fluorescence, or proteins that modify the neuronal population under study. However, genetic killing, electrical silencing or blocking of synaptic neurotransmission may produce compensatory effects if activated during development. By choosing a promoter with late onset of expression, these unwanted side effects can be avoided. An alternative is to use an inducible version of the Cre protein, modified to translocate to the nucleus upon addition of estrogen analogs and only then able to exert its recombination activity on lox sites (131).

3.3.2. Removal of neurons or their activities

A direct approach to test the functionality of a specific set of neurons participating in a neuronal circuit is to kill the neurons. Genetic elimination of neurons is usually achieved by expression of the diphtheria toxin A subunit (DTA; (132), which blocks protein synthesis vital for survival. A more advanced system including temporal control of the ablation is based on Cre-dependent expression of a diphtheria toxin receptor (DTR) in mouse cells and the subsequent application of the diphtheria toxin (DT). The toxin needs to get inside cells to exert its toxicity and only cells that carry the DT receptor will be sensitive to the diphtheria toxin. The toxin can therefore be handily injected intraperitoneal and since the diphtheria toxin crosses the blood-brain barrier, cell ablation is also possible in the central nervous system (133). While this approach is elegant in principle, few successful studies have been reported. Also, overexpression of channels can effectively silence neuronal activity (134) but potentially, such strategies may induce apoptotic cell death in neurons (135). An alternative approach to silence neurons is to use methods based on toxins. For example, expression of the tetanus toxin light chain (TeTxLc) will block action potentials and synaptic release through cleavage of the synaptic protein synaptobrevin (136-138). In the mammalian spinal cord, specific inactivation of synaptic activity of the V3 neurons by use of a reporter TeTxLc/TeNT mouse has been reported from the Goulding laboratory (53). Another useful technique to acutely target locomotor activities, employs the aforementioned allatostatin receptor, that upon delivery of allatostatin, silences neurotransmission. In spinal cord analysis, the Drosophila allatostatin receptor (AlstR) was expressed in V1 spinal interneurons by use of Engrailed-1Cre mice. By the addition of allatostatin to spinal cord in-vitro preparations from such mice, the activity of V1 interneurons activity was decreased and the locomotor rhythm slowed (62). This has also been tested in-vivo, where mice expressing AlstR in V3 neurons received allatostatin intrathecally resulting in increased variation of step cycle length (53). A non-invasive approach to neuronal activity control can be achieved using the DREADD (designer receptors exclusively activated by designer drugs) technology. The DREADD permits selective activation of receptors in a genetically targeted population of neurons. The ligands can be injected peripherally and cross the blood-brain barrier to activate the receptors making this tool useful for studying the connection between activity of a particular receptor and the underlying behaviour (Alexander at al. 2009, Neuron).

3.3.3. Observing neuronal activity

In the analysis of neuronal network functionality, it is desirable to detect membrane voltages or signals consequential of electric activity such as free calcium, cyclic nucleotide concentrations or pH. Various genetically encoded probes have been generated using fluorescent proteins (139, 140), most of them variants of green fluorescent protein (GFP). The selective introduction of genetically encoded probes into neurons eliminates disturbing signals from irrelevant neuronal populations and glial cells.

Intracellular free calcium concentrations determine neuronal function both through regulation of ion channels and through changes of gene expression. Neurotransmitter release from synaptic vesicles is triggered by an elevation of calcium (141, 142). The most common Ca2+-binding motif in mammalian genomes is in the ubiquitous Ca2+-sensor protein calmodulin (143). The first protein based calcium sensors were constructed as fusion proteins of calmodulin and two GFP variants where the detectable signal was induced by a conformational change of calmodulin that in turn induced FRET (144, 145). Several other classes of fluorescent Ca2+ indicator probes are now available, most of them based on calmodulin, for example an improved GCaMP variant. GCaMP3 was reported brighter with greater stability and dynamic range as well as an improved sensitivity in brain slices and was successfully adenovirus-delivered to image the *in vivo* calcium activity of motor cortical neurons (146). However, reporter mice expressing efficient calcium reporters are not yet available. Bulk loading of calcium indicators like fura-3 and Oregon-BAPTA is a common and reliable alternative method to measure neuronal activity, which can be easily combined with genetic labeling of neuronal subpopulations.

To measure single fast action potentials and sub-threshold activities of neurons using genetic approaches, it is necessary to identify proteins capable of sensing voltage fluctuations. This has proven to be rather challenging. Voltage-gated ion channels undergo conformational changes over a narrow voltage range, which restricts the response range and in addition, GFP voltage sensors seem to get trapped in internal compartments. Nevertheless, first generation voltage sensors has been produced by fusing GFP to the sixth trans-membrane domain of a mutated version of the Shaker potassium channel (147, 148) or by

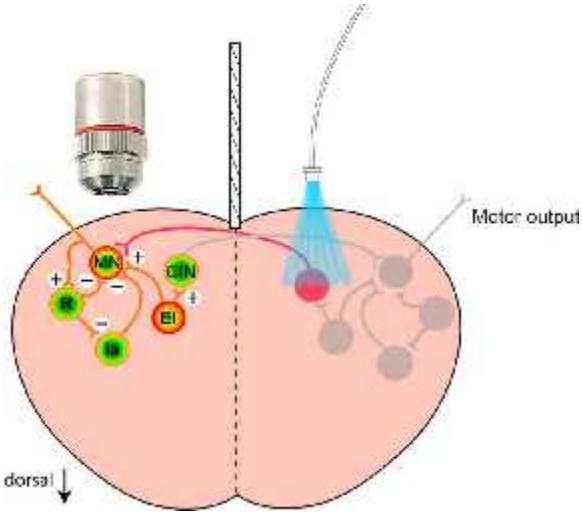

Figure 2. Schematic outline for an experimental set-up combining ipsilateral optogenetic activation of commissural interneurons (CINs) with 2-photon imaging of contralateral network activity. Contralateral CINs (red), which have been modified to express channelrhodopsins, are activated by laser light, either in the native state or during locomotion. On the ipsilateral side, cells have been bulk-loaded by a suitable indicator of activity (Oregon-BAPTA, Fura-3). This can be further combined with genetic labeling of subpopulation through known genetic markers. Activities can then be correlated after direct stimulation of CINs and to those recorded during fictive locomotion.

fluorescence resonance energy transfer (FRET) between cyan and yellow emitting fluorescent proteins (CFP and YFP) linked in tandem and fused to a truncated potassium channel (149). In the last decade, several groups have made progress on this task and have reported on the development of novel fluorescent proteins as well as their use in the construction of voltage-sensitive fluorescent proteins (150). For example, second generation voltage sensors have been built on the Ciona voltage sensor–containing phosphatase where the enzyme domain is replaced with two fluorescent proteins to proved FRET upon voltage changes (151). When voltage sensors of this efficiency can be targeted to specific cell populations by use of a reporter mouse line combined with Cre-lines (or virus delivery), prospects for circuit analysis indeed shine bright.

3.3.4. Activation of neurons

Electrophysiological experiments only allow a small number of neuronal contacts to be analyzed simultaneously, which makes analysis of homogenous neuronal populations involved in neuronal circuit function time consuming. Genetic approaches can be used to circumvent this problem. To achieve faster and more precise control of stimulation in functional analyses of networks, techniques for depolarizing neurons using light in combination with genetic approaches are now used. Such light-induced stimulation would momentarily hijack the control of neurons from a circuit without destroying their connections. In 2005, it was reported that introduction of a microbial opsin gene could render neurons to become precisely responsive to light (152). Several versions of opsin proteins including channelrhodopsin and halorhodopsin have now been demonstrated to capable of taking control of neurons in response to light (153). At the moment Cre recombinase–dependent opsin-expressing viruses is probably the most efficient way to deliver these with the extensive and growing resource of mouse lines selectively expressing Cre recombinase in defined cell types; optogenetic control can now be delivered to defined cells in freely moving mice with substantial versatility (154). Together with the possibility of observing neuronal circuits in living organisms, it is now tractable to stimulate one part of the circuitry and visualize the response in the remaining components. For CINs, this would allow for ipsilateral stimulation with light while detecting contralateral CPG and motor neuron activities (Figure 2).

4. FUTURE DIRECTIONS AND UNANSWERED QUESTIONS

Here we have reviewed the current knowledge regarding neuronal populations participating in spinal cord left-right communication including their effect on the coordination of the CPG. New technology together with added knowledge about these circuits in combination with the accessibility and ease of analysis are great assets in the continuing quest to reach a thorough understanding of a CPG network, and in particular the role of CINs. Many questions remain to be answered. How many functional subpopulations of spinal CINs are there? Can we define a subpopulation of inhibitory CINs responsible for left-right alternation? Can we define a subpopulation of excitatory CINs responsible for left-right synchrony? Can subtypes of CINs be associated to subclasses of ipsilateral INs or MNs? Are CINs multitaskers? Are different CIN subtypes reliant on different sets of midline axon guidance cues? With growing knowledge and new emerging tools, these questions could soon find their answers.

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