Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Neurons, Circuitry, and Plasticity in the Spinal Cord and Brainstem*

Dorsally derived spinal interneurons in locomotor circuits

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During neuronal circuit formation, axons are guided to their targets by the help of axon guidance molecules, which are required for establishing functional circuits. A promising system to dissect the development and functionalities of neuronal circuitry is the spinal cord central pattern generator (CPG) for locomotion, which converts a tonic supraspinal drive to rhythmic and coordinated movements. Here we describe concepts arising from genetic studies of the locomotor network with a focus on the position and roles of commissural interneurons. In particular, this involves studies of several families of axon guidance molecules relevant for midline crossing, the Eph/ephrins and Netrin/DCC. Effects on developing commissural interneurons in mice with aberrant midline axon guidance capabilities suggest that, in addition to ventral populations, dorsal commissural interneurons also play a role in coordinating locomotor circuitry. Recent findings implicate the novel dI6 interneuron marker Dmrt3 in this role. Strikingly, mutations in Dmrt3 result in divergent gait patterns in both mice and horses.

Keywords: neuronal network; spinal cord; central pattern generator; Dmrt3; netrin; Eph

Introduction

Locomotor central pattern generators (CPGs) in the spinal cord are neuronal networks that produce rhythmic activities necessary for coordinated trunk and limb movements.[1–5] The CPGs are mainly responsible for the generation of a stable rhythm while at the same time coordinating flexion extension of limbs and alternation of movement between the right and left sides of the body. Left–right locomotor coordination requires commissural interneurons (CINs) defined by their projections to the contralateral side of the spinal cord. In mammals, many CINs are rhythmically active during locomotor-like activity, and both excitatory and inhibitory CINs are considered to coordinate left–right activities during locomotion.[6–9] Lesion experiments in neonatal spinal cords have shown that the dorsal spinal cord is dispensable for rhythmic and coordinated locomotor activity[10] and that normal left–right alternating locomotion disappears after cutting the ventral commissure. These experiments suggest that basic left–right coordination is mediated by ventrally located CINs, at least in the perinatal rat spinal cord. Mouse mutants that display aberrant axon guidance over the midline are useful to analyze the contribution of neuronal subpopulations to left–right regulation of locomotor CPG activity. Such studies have raised a possible role for dorsally originating interneurons in locomotor coordination.

Studies aimed at identifying the subcomponents of the locomotor CPG, as well as ancillary components responsible for left–right coordination, are challenging but nevertheless critical to dissecting the development and function of locomotor neuronal circuitry. Subpopulations of spinal cord interneurons can be identified during development by their expression of specific homeodomain transcription factors.[11] 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 produce CINs.[12–17] Out of the ventral subtypes, the V0 and V3 populations are considered to generate CINs and have been investigated in genetic studies on left–right locomotor coordination. During development some dorsally born neurons migrate ventrally,[12–14,16]

doi: 10.1111/j.1749-6632.2012.06801.x

Ann. N.Y. Acad. Sci. 1279 (2013) 32–42

suggesting that neurons originating from the dorsal spinal cord might participate in ventral-located circuitries. Moreover, dorsal interneurons are activated during locomotion as demonstrated by c-Fos activation.[18,19] Indeed, dorsally originating interneuron populations also extend commissural projections,[17,20–22] and project directly to motor neurons[23] and are therefore candidates regulating midline coordination during locomotion.

Netrin-dependent interneurons

During spinal cord development, differentiating commissural interneurons send their axons toward and across the floor plate to form the ventral commissure. A set of axon guidance molecules, the Netrins, acts as diffusible floor plate chemotrophic guidance cues for commissural axons in the mouse spinal cord.[24] Netrins bind to and activate the Netrin receptor DCC, which mediates an attractive effect to the growing axons, and in Netrin-1 knockout mice, the majority of dorsal commissural interneurons no longer find and cross the midline.[25] Given their important role in guiding neurons toward the midline, Netrin-1 and DCC are obvious candidates for providing guidance cues to commissural interneurons involved in CPG left–right coordination (Fig. 1A). Indeed, formation of an alternating locomotor CPG network requires the presence of Netrin protein as loss of Netrin-1 results in complete synchrony between the two sides during fictive locomotion.[17] Therefore, Netrin-1 and DCC mouse mutants were used to address the role of commissural interneuron subpopulations in left–right coordination.

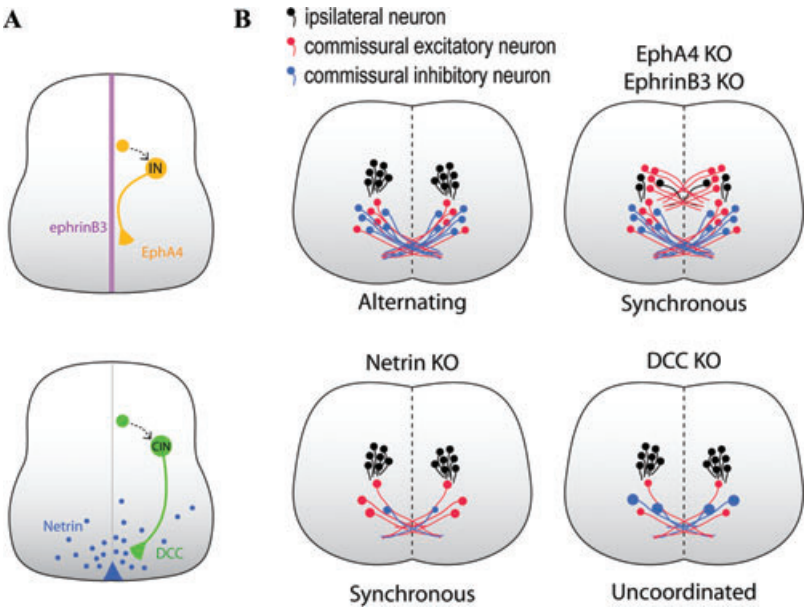

Figure 1. Schematic models of CPG networks in axon guidance mutant animals. (A) EphA4/ephrinB3 and Netrin-1/DCC have mirror roles in the guiding of axons away or toward the midline. While EphA4 prevents axon fibers from crossing the midline and remaining ipsilateral, Netrin attracts fibers to the midline. (B) Consequently, *EphA4/ephrinB3* and *Netrin-1/DCC* mutant mice differ in their shaping of locomotor circuitry. Under normal conditions (upper left), excitatory and inhibitory CINs are balanced to produce alternating left/right CPG activities. In EphA4- and ephrinB3-null mutants, aberrant midline crossing of ipsilateral interneurons shifted the balance between excitation and inhibition, leading to synchronous left/right CPG output. In Netrin-1-null mutants, reduced midline crossing of fibers also led to a similar shift in the balance between excitation and inhibition but was due to reduced number of inhibitory CINs. Again, this resulted in a synchronous output, but in contrast to EphA4/ephrinB3-null mutants, the synchrony is not reversible by pharmacological strengthening of inhibition (not shown). This might be explained by a too limited number of commissural inhibitory fibers left in *Netrin*-1-null mutants in which the drug can be active. We hypothesize that under normal conditions, inhibitory connections dominate with alternation as a result. When excitatory action over the midline is strengthened, as in the *EphA4*-null mice, or when inhibitory action over the midline is weakened, as in the *Netrin-1*-null mutant mice, the excitatory contralateral component takes over, leading to coupling of the two spinal cord half centers and synchrony of the rhythmic output. Finally, in DCC-null mutants, a decreased midline crossing led to a severe reduction of both excitatory and inhibitory fibers, resulting in a complete collapse of left–right coordination.

The number of CINs and fibers crossing the spinal cord midline are strongly reduced in mouse mutants lacking *Netrin-1* (Fig. 1B, Refs. 17 and 25). Since Netrin-1 is a potential attractant for fibers from cells originating from both dorsal and ventral neuroepithelium, it has been unclear which neuronal subpopulations and functions are dependent of Netrin-mediated axon guidance. Commissural interneurons are found within the dI1-dI3, dI5, dI6, V0, and V3 subpopulations of neurons. Of these, dI1–dI3, dI5, dI6, and V0d cells were most severely affected in *Netrin-1* mutant mice, having a 75–80% reduction of commissural traceable axons, indicating a strong dependence on Netrin-1 to properly find the midline. Interestingly, the ventral-most population (V3) was completely unaffected.

There are approximately twice as many inhibitory CIN terminals than excitatory ones in both rat and mouse spinal cords.[17,26] In Netrin-1 mutants, a greater number of inhibitory than excitatory CINs are lost, resulting in the converse situation—a prevailing majority of excitatory CINs. Since the remaining coordination is synchronous, excitatory CIN action is presumably more important for general cross-midline activation than for maintenance of left–right alternation. Furthermore, since mice that, in principle, lack excitatory activity in the spinal cord through loss of VGLUT2 did not show any locomotor rhythm or coordination deficiencies,[27–29] inhibitory action is sufficient to produce normal rhythm and coordination. Inhibitory commissural connections have previously been suggested to be major constituents of left–right phasing during locomotion,[8,14,30–34] and in cats, such interneurons have been implicated in mediation of the crossed reflexes and in the selection of different motor patterns.[35,36] Functional roles for Netrin-1–guided commissural interneurons in the spinal cord are evident, and together with the fact that Netrin-1 preferentially attracts dorsally originating CINs of an inhibitory character to the floorplate, we should consider those as potential populations regulating left/right alternation.

Lack of DCC results in collapsed left–right coordination

Mice that carry a null mutation of DCC display a severe loss of interneuronal subpopulations originating from commissural progenitors as well as matured commissural interneurons.[37] The loss of CINs is accompanied by completely uncoordinated left–right ventral root activities during fictive locomotion (see schematic in Fig. 1B). Thus, DCC plays a crucial role in the formation of spinal neuronal circuitry coordinating left–right activities. Of note, similar to Netrin-1 mutants, flexion-extension ventral root activities remained alternating. The loss of commissural fibers from V3 neurons in mice lacking DCC was the only significant difference compared to Netrin-1 mutant mice, and resulted in a complete loss of coordination between the left and right side, emphasizing the fundamental role of V3 CINs in coordinating synchronous activities over the midline. Taken together, in mice lacking DCC, a limited number of CINs crossed the midline, which functionally resulted in uncoordinated left–right activity during fictive locomotion. This severe reduction of CINs did not shift the balance between excitatory and inhibitory fibers over the midline, likely due to an almost similar loss of CINs originating from different progenitor domains. In summary, while Netrin and DCC mutant mice have a similar severe reduction of CINs, their fictive locomotion phenotypes are markedly different, producing synchronous versus uncoordinated patterns of activation (see schematic in Fig. 1B). Thus, axon guidance mediated by Netrin and DCC is required to form a circuitry capable of coordinating left–right activities.

Lessons from Eph and ephrins

Previous studies have shown that the axon guidance molecules EphA4 and ephrinB3 cooperate to prevent ipsilateral interneurons from crossing the midline in the spinal cord and if either molecule is deleted in mice, this will result in a synchronous hopping gait.[38] To further investigate the origin of these interneurons, we generated a conditional allele targeting the third exon of *EphA4* (EphA4lox; Supporting Fig. S1), which was found to delete the EphA4 protein in a spatially restricted manner.[39] In addition, mice with a complete deletion of *EphA4,* produced by crossing *EphA4lox* mice with a general Cre deleter (PGK-Cre; *EphA4lox/lox),* displayed all characteristics of regular EphA4-null mutants, including a synchronous gait.[39,40] To achieve restricted deletion of EphA4 in the dorsoventral axis of the lumbar spinal cord (Fig. 2A and B), we crossbred the conditional *EphA4* allele to mice expressing

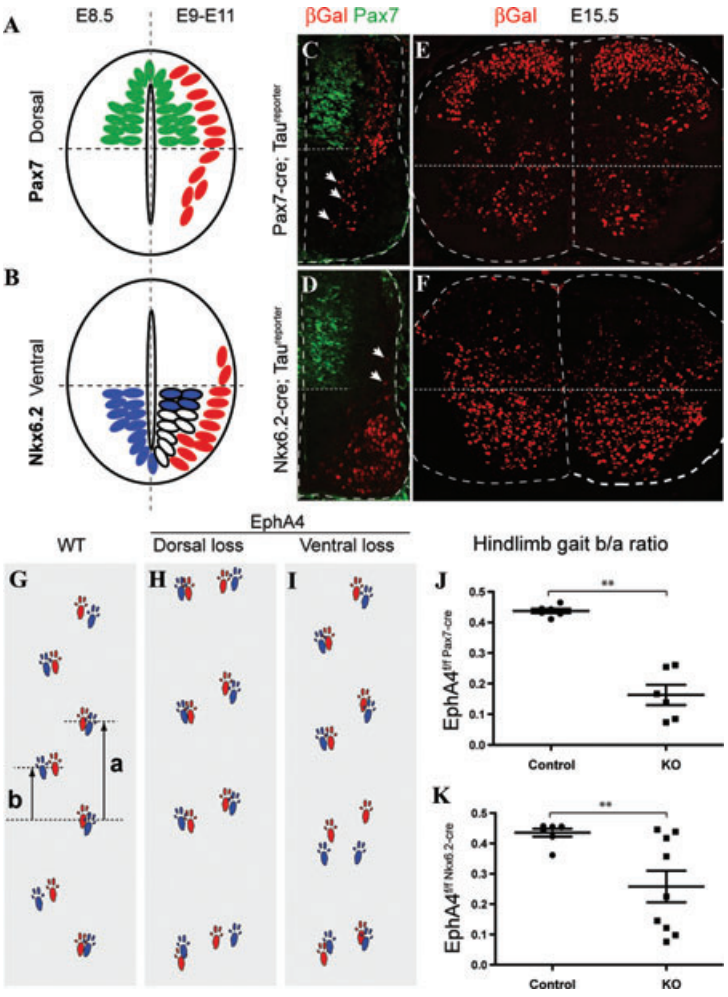

Figure 2. Deletion of EphA4 in dorsal progenitor cells leads to near synchronous gait. (A and B) Schematic outline of Pax7 and Nkx6.2 expression in cells of the developing spinal cord. Pax7-expressing dorsal progenitors in the neural tube[78,79] are labeled in green. At e8.5, Nkx6.2 is expressed in a broad ventral domain of the mouse neural tube (blue). At later stages (e9.5–e11.5), Nkx6.2 expression is largely confined to a narrow domain immediately ventral of the horizontal midline (blue).[70] Note that cells in the entire ventral domain (black circles) have been exposed to Cre at e8.5 and are therefore subsequently detectable when these cells mature into neurons (red). (C–F) Analysis of Pax7-Cre and Nkx6.2-Cre–mediated recombination. Pax7-Cre and Nkx6.2-Cre mice were crossed with $Tau^{mEGFP,NlsLacZ}$ reporter mice and β-Gal expression in e11.5 (C and D) and e15.5 (E and F) embryos was analyzed. At e11.5, costaining of Pax7 and β-Gal in Pax7-Cre/$Tau^{mEGFP,NlsLacZ}$ embryos display predominantly dorsal expression of β-Gal (C). When cells originating from Pax7 progenitors start to differentiate into neurons, Pax7 is downregulated and the expression of β-Gal driven from the promoter of the axonal microtubule-associated protein tau is initiated. This enables detection of neurons during their radial and/or ventral migration from the Pax7 mediodorsal compartment between e9.5 and e11.5 (red in A and B). Some expression in ventrally located cells is detected (arrows), largely corresponding to the earliest born dorsal neurons that migrate ventrally. Nkx6.2-Cre/$Tau^{mEGFP,NlsLacZ}$ embryos at e11.5 show an extensive and strong expression in ventrally located neurons together with a weak expression in a few cells that appear to be migrating ventrally from dorsal positions (arrows, D). At e15.5, the expression in β-Gal in Pax7-Cre/$Tau^{mEGFP,NlsLacZ}$ spinal cords is restricted to dorsal lamina and to scattered ventral and intermediate cells (E). In Nkx6.2-Cre/$Tau^{mEGFP,NlsLacZ}$ spinal cords, the expression of β-Gal is widespread in ventral cells and is present in some cells that are more dorsally located (F). Weak dotted lines indicate the boundary between dorsal and ventral horns. (G–K) Gait analyses of WT, $EphA4^{f/fPax7-cre}$ (H) and $EphA4^{f/fNkx6.2-cre}$ mice (I). EphA4 conditional knockout mice were generated by homologous recombination in embryonic stem cells, essentially as described previously.[40] Forepaws (red) and hindpaws (blue) were painted and the distance between left and right paws (b in G) and distance covered by the same paw (a in G) was determined. Comparison of the b/a ratio of wild-type ($n = 6$) and $EphA4^{f/fPax7-cre}$ mice ($n = 6$) show a pronounced decrease in all mutant mice analyzed (E). The b/a ratio of $EphA4^{f/fNkx6.2-cre}$ mice ($n = 8$) compared to Wt ($n = 8$) show a variable phenotype with some animals displaying a close to normal gait pattern while some are more affected (F).

Cre in the dorsal (Pax7-Cre[41]) or ventral progenitor domain of the spinal cord (Nkx6.2-Cre[42]). The specificities of the two Cre alleles were validated using *TaumGFP-nlslacZ* reporter mice,[43] followed by histochemical stains with antibodies against β-galactosidase and Pax7. At E11.5, the dorsoventral specificities are clearly recognized, whereas at E15.5, the pattern of lacZ expression suggests that some of the neurons born in the Pax7 expression domain migrate ventrally (Fig. 2C and E). Similarly, but less pronounced, some of the neurons born in the Nkx6.2 domain have a tendency to expand dorsally (Fig. 2D and F). Whereas neonatal fictive locomotion was normal, gait analysis demonstrated that the walking pattern of *Pax7-Cre; EphA4lox/lox* animals markedly differed from control animals (Fig. 2G–K). The b/a ratio, indicating the stride distance between the left and right hindpaw, was close to 0.5 for controls (including *EphA4lox/lox* animals), while it was significantly lower (0.15) for *Pax7-Cre; EphA4lox/lox* mice (Fig. 2J). The b/a ratio in *Nkx6.2-Cre; EphA4lox/lox* mice was also significantly affected, although with an average value of 0.25, suggesting a lesser influence on locomotor coordination from EphA4-dependent neurons originating from the ventral domain (Fig. 2K).

This genetic deletion of *EphA4* in the dorsal or ventral domain of the spinal cord revealed that mice affected in the dorsal subpopulations produce close to a synchronous gait. Mice with an *EphA4* deletion in the ventral spinal cord were also affected, but to a lesser degree. This suggests that dorsally originating ipsilateral interneurons, when misdirected, are interfere with normal locomotion. Thus, subtype-specific deletion of EphA4 in dorsal spinal cord populations demonstrated a stronger influence on left–right coordination compared with deletion in ventral populations. Likewise, studies of DCC and Netrin axon guidance molecules suggest that dorsal populations play a role in coordination of locomotion. Neurons originating from dorsal progenitors are at least partly responsible for the phenotype observed in $EphA4^{-/-}$ and $Ntn1^{-/-}$ mice, emphasizing the importance of investigating these populations in relation to locomotor network coordination.

Location and function of locomotor interneurons

V0 interneurons arise from p0 progenitor cells expressing the Dbx1 homeodomain (HD) protein and consist of two populations of cells, one of which expresses the HD protein Evx1.[12,15,16] When leaving the proliferative zone, these cells take on a ventral migratory route to settle in lamina VII/VIII and extend their axons rostrally on the contralateral side of the spinal cord. Dbx1 is expressed in progenitor cells giving rise to both V0V and V0D neurons and the loss of both V0V and V0D in Dbx1-knockout mice led to intermittent episodes of synchrony between left and right ventral roots during fictive locomotion. Sim1-expressing V3 neurons, which arise from Nkx2.2-positive p3 progenitors, are predominantly commissural excitatory and part of this population appears to settle close to their origin in lamina VIII.[44] Silencing of the V3 population using Sim-Cre/TeNT resulted in an irregular and imbalanced motor rhythm.[45]

Although ipsilateral populations of neurons do not directly regulate 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.[46,47] For this reason, genetic studies of specific ipsilateral subpopulations with regard to their function during locomotion are informative of left–right activities. V1 progenitors expressing HD proteins Dbx2 and Nkx6.2 give rise to Engrailed 1 (En1)-positive V1 interneurons.[48] These cells migrate to a ventrolateral position in lamina VII where they develop local projections.[49] Studies of En1/2-expressing V1 interneurons have shown that in "simpler" vertebrates, such as the fish[50] and frog,[51] these neurons represent a homogenous cell population of ipsilateral glycinergic inhibitory interneurons that play important roles in motor control and sensory gating during swimming, while they appear to have more heterogeneous functions in higher vertebrates.[52–54] 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.[55] 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.[56,57] 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[46] during fictive locomotion and locomotion in adult mice show a speed-dependent loss of left–right alternation defined by a transition to synchronous gait at high speed.[58] 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)[49] or drifting in and out of strict alternation (V2a).[14] Consequently, it seems likely that multiple neuronal subtypes originating from several ventral progenitor domains are involved in the different aspects of left–right coordination.

Of the dorsal IN populations, commissural inhibitory dI6 neurons, which settle in laminae 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.[14,30–33] Moreover, dI6 neurons share some properties with dorsal V0 interneurons in that they originate from progenitors with similar character and migrate along similar routes.[14,17] Interestingly, one subset of dI6 neurons have been demonstrated to phase-lock with ventral root outputs, which is in concert with a role to coordinate motor output during locomotion.[59] In the same study, another subset was demonstrated to oscillate intrinsically when isolated from excitatory input, potentially indicating an involvement in locomotor rhythm generation. Possible roles for dI4 and dI5 interneurons for CPG coordination remains to be determined; notably, however, a cohort of dI4 neurons have been reported to form contacts on Ia afferent terminals near MNs.[60] Interestingly, dI3 neurons have been demonstrated to directly contact MNs by rabies virus tracing[61] and Isl1 positive dI3 cells, which migrate ventrally to lamina VII, receive direct primary afferent inputs as well as rhythmic inputs, suggesting a role for these dI3 neurons in sensorimotor integration.[62] Finally, dI1-dI2 interneurons are less likely to contribute to CPG coordination, since they have been suggested as part of ascending pathways, including the spinocerebellar and the spinothalamic tract.[12,13] In any case, multiple lines of evidence suggest that neurons from the dorsal neuroepithelium are also likely to contribute to locomotor coordination.

A novel dI6 marker having a role for gait

In a search for genes expressed predominantly in cholinergic cells located in the P11 ventral spinal cord, we found *Dmrt3*.[63] Another study using a similar screen strategy also identified Dmrt3 as a marker for a subset of spinal cord neurons.[64]The DMRT family consists of three members (1–3), which carry a DM (dsx and mab-3) DNA-binding domain conferring sequence-specific DNA binding distinct from a classical zinc finger.[65] Dmrt3, the least characterized of the three members, is expressed in neural and germ cells and has been associated with sexual development.[66] At postnatal stages, Dmrt3 is expressed in the ventral spinal cord while at earlier time points, expression was evident in more dorsally located cells, indicative of dorsal to ventral migration.[67] The origin of the Dmrt3 cells was characterized using markers for dorsal and ventral progenitors and found to be a subset of dI6 neurons (Fig. 3A). At E12.5 and E14.5, there was also a partial overlap with neurons positive for the Wilms tumor 1 (WT1) protein, which has been suggested to label a dI6 population.[68] Retrograde tracing experiments demonstrated that Dmrt3 interneurons extend projections both ipsi- and contralateral and transsynaptic pseudorabies virus-based tracing demonstrated direct connections to motor neurons both ipsi- and contralateral to the injected muscle (schematized in Fig. 3B). Finally, Dmrt3 interneurons are VIAAT-positive/VGLUT2-negative suggesting that they are inhibitory.

Independent of these efforts, in a genome-wide association study (GWAS), Leif Andersson and his coworkers identified a region of the chromosome associated with the ability to pace in Icelandic horses. They explored the circumstance that some Icelandic horses are four-gaited, while some are five-gaited having the additional ability to pace at high speed, so called flying pace. The identified region spanned four genes; and when revealed to us, we informed the Andersson group of our cellular data. Thus, even if pure positional cloning would have identified the mutation, it was clear that of the four genes, *Dmrt3* was the obvious candidate. Sequencing of the *Dmrt3* gene in pace and a nonpace Icelandic horses soon thereafter identified a nonsense mutation in the *Dmrt3* gene, verifying the GWAS result. The mutation is a single nucleotide substitution that leads to a premature stop codon and expression of a truncated

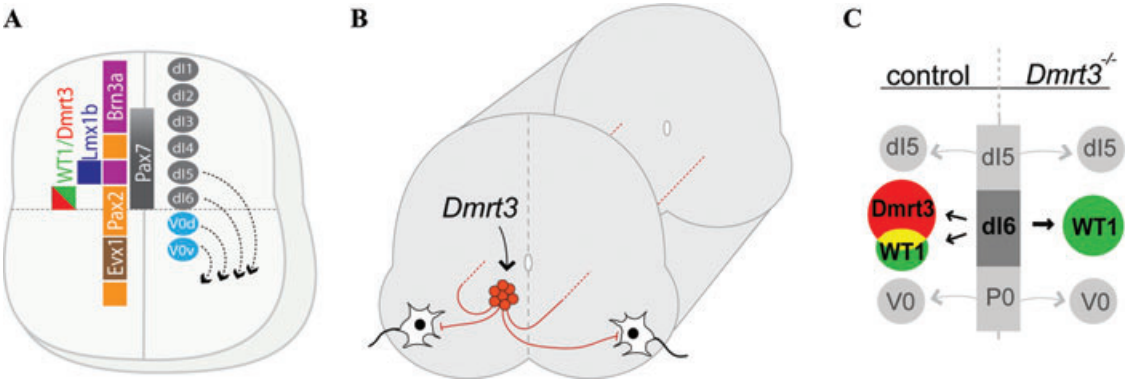

Figure 3. Characteristics of the Dmrt3 spinal cell population. (A) Schematic spinal cord cross section showing progenitor and transcription factor domains. Dmrt3$^+$ cells originate from the ventral-most part of the dorsal domain (border indicated by line). Dmrt3$^+$ cells overlap with the dI4/dI6/V0d marker Pax2 but not with the $V0_V/V0_C/V0_G$ marker Evx1 or the dI5 marker Lmx1b. Dmrt3 and WT1 show a partial overlap. (B) Schematic of position and demonstrated possible projections of Dmrt3 neurons in the mouse spinal cord. (C) Schematic illustration of fate change in the Dmrt3 dI6 population of neurons.

version of Dmrt3.[67] The mutation is present on both alleles in pacing Icelandic horses and has a major effect on the pattern of locomotion in horses. Domestic horses with the ability to perform alternate gaits at intermediate speeds, possessing toelt, classicofino, and fox trot are all homozygous for the Dmrt3 mutation, while nongaited horses are homozygous for the wild-type allele. Moreover, the Dmrt3 mutation has accumulated in harness race horses in which the transition from trot to gallop leads to disqualification from the race.[67]

We analyzed a possible role for Dmrt3 in dI6 population development in *Dmrt3*-null mutant mice. These mice have a shorter lifespan and occasional male sexual developmental abnormalities.[69] In general, loss of transcription factor expression within spinal cord progenitor domains results in specification defects in the produced neurons, presumably by suppression of differentiation programs that operate in adjacent domains.[68] For example, in *Dbx1* mutant mice, progenitors that give rise to V0 interneurons instead generate V1 and dI6 interneurons,[14,16] and loss of the V1-specific *Nkx6.2* homeobox transcription factor results in increased numbers of V0 interneurons at the expense of V1s.[70] The dI6 population, as well as the flanking dI5 and V0d populations, remained of normal sizes in *Dmrt3*$^{-/-}$ mice.[67] In contrast, the number of WT1$^+$ neurons increased, while the number of commissural interneurons decreased, likely explained by an altered fate of the Dmrt3 neuron population (Fig. 3C).

Drug-induced fictive locomotion in Dmrt3 mutant mice displayed a strikingly uncoordinated and irregular firing rhythm both between the left–right and flexion–extension outputs. Moreover, airstepping in 1- and 4-day-old mice was also severely disturbed. Despite this very irregular motor output, walking abnormalities in adult knockout mice were not previously reported.[69] A closer examination on a treadmill revealed an inability of Dmrt3 mutant mice to run at higher velocities. At a speed where gait recordings were possible, significant increases in stance and swing times were apparent.[67] Dmrt3$^+$ cells are inhibitory and can have ipsi- and/or contralateral projections, and since previous studies have suggested that inhibitory commissural connections are major constituents responsible for left–right phasing during locomotion,[71–73] a role for Dmrt3 in left–right coordination was envisaged. Interestingly, in Dmrt3 mutant mice, left–right as well as flexion–extension perinatal locomotion collapses, and of note, this is the first example of a mouse mutant with an ipsilateral (flexion-extension) coordination failure. Yet, the locomotor coordination abnormalities in adults were relatively mild and confined to changes in flexion-extension circuitry, suggesting a remarkable adaptability in locomotor circuit development and function. The observation of an extensive flexibility of spinal cord circuitry has been reported previously.[74] While the most likely explanation for the observed defects lies in the changed fate within the dI6 neuron population, the extent of circuit reorganization is not known and requires

further investigation, such as the determination of the physiological character of the Dmrt3 population, the role of the increased WT1 population, possible regulation of other transcription factors, and effects on other possible dI6 subpopulations.

In quadrupedal mammalian locomotion, speed regulation is accompanied by a change of gait. Interneuron populations are recruited to locomotor coordination depending on running speed while in zebrafish, the recruitment of spinal interneurons at higher speeds results in silencing of interneurons recruited at lower speeds.[75] In mice, an increase in running speed leads to a gradual recruitment of interneurons[76] and such involvement of V2a interneurons is required for the maintenance of left–right alternation at escalating velocities.[58] In the same way, Dmrt3 dI6 neurons may have a similar role in the flexion-extension cycle. At lower velocities, the loss of Dmrt3 does not seem to have a large effect, while at intermediate running speeds, *Dmrt3*-null mice increase their stride length, and finally, they refuse to run at higher speeds. This suggests a role of Dmrt3 in maintaining strict and swift flexion-extension alternation.

Despite the differences in speed and size between mice and horses, the phenotypic dependencies downstream of Dmrt3 are relatively comparable. Running at higher speeds is characterized by shortening the extensor phase, while the flexor phase is largely unchanged.[77] An increased stride length, which was observed in *Dmrt3*-null mice, may thus be a selectable advantage for harness racing horses. Interestingly, harness racing horses are not able to keep up with nonharness racing horses during gallop, which complements the finding that Dmrt3-null mice give up at higher treadmill speeds. The truncated Dmrt3 allows horses to move beyond its three natural gaits and to perform alternative gaits. In the mouse, lack of Dmrt3 results in impaired limb coordination in newborns and in adults it affects their ability to run at higher speeds. Thus, the loss of Dmrt3 results in a more permissive and flexible state of the locomotor circuit. This suggests that Dmrt3-positive neurons might be important for maintaining precise flexion-extension alternation. Taken together, a role for Dmrt3 neurons in coordinating both left–right alternation and flexor–extensor motor neuron activity seems plausible.

Our data demonstrate that Dmrt3 cells serve as both contralateral and ipsilateral neurons, and the complete collapse of early CPG coordination opens for speculative thoughts regarding the role of these neurons in organizing spinal output. To us, the most parsimonious explanation positions Dmrt3 neurons as coordinators between left–right and flexion–extension activities. We hypothesize that these neurons are required for the early CPG coordination circuitry, and in adults, they may act as phase-lock neurons to control and secure a robust gait. Interestingly, the observed flexibility of the developing spinal cord circuitry suggests that alternative circuitries are shaped in the absence of Dmrt3. Neurons, which replace the role of Dmrt3-dependent neurons, are clearly able to create left–right as well as flexion–extension activities, however, not with the same restraining action in adults. Presumably, the contra- and ipsilateral projections of the Dmrt3 neurons are unique features among locomotor circuit neurons and cannot be replaced entirely. The fact that Dmrt3 neurons are all inhibitory implies that they are positioned so that flexion activates Dmrt3 neurons, which results in inhibition of ipsilateral extension and contralateral flexion activities.

In conclusion, Dmrt3 is specifically expressed in the dI6 subdivision of spinal cord neurons, takes part in neuronal specification within this subdivision, and is critical for the development of a coordinated locomotor network with emphasis on the regulation of swing and stance phases in the mouse. Dmrt3 interneurons project fibers ipsi- and contralaterally, which together with a significant phenotype in flexor-extensor coordination in fictive locomotion, positions Dmrt3 in a pivotal role for configuring the spinal circuits controlling stride. The discovery of a previously unknown molecule required for shaping a neuronal ensemble coordinating limb movement in at least two species, and with factual relevance for horse locomotion, will have an impact on our understanding of gait control and the underlying flexibility in neuronal circuit hardwiring. Further studies of the Dmrt3 neurons will be necessary to define the exact role of these neurons in the locomotor network. Given the results from studies of axon guidance molecules and the evident important role for dI6 neurons, we conclude that dorsally derived spinal interneurons indeed play a significant role in locomotor circuits.

Acknowledgments

We thank M. Tessier-Lavigne (Netrin-1), E. Andersson and J. Ericson (Nkx6.2-Cre), and M. Capecchi (Pax7-Cre) for providing mice, and Dr. T. Boesl for the generation of EphA4 transgenic mice. Work in the lab is supported by grants from the Swedish Research Council—Medicine and Health, the Swedish Brain Foundation, the Hållsten Foundation, the Knut and Alice Wallenberg Foundation, and Uppsala University. K.K. is a Royal Swedish Academy of Sciences Research Fellow supported by a grant from the Knut and Alice Wallenberg Foundation.

Supporting Information

Additional supporting information may be found in the online version of this paper:

Figure S1. Design and production of an EphA4 conditional allele. (A) Schematic overview of the construct used to target genomic DNA in ES cells. A cDNA encoding EphA4 from exon IV and onward and a polyA (pA) tail was fused to exon III with 5′ genomic sequence remaining intact. LoxP sites were inserted before and after the exonIII-cDNA. After the second loxP site, exon III with its splice acceptor site was inserted to allow production of an alternative transcript after excision of the EphA4 cDNA fusion. An internal ribosome entry site (IRES) sequence was placed before a lacZ-TTC reporter construct for production of retrogradely transported β-gal protein. Finally, a Frt-flanked Neo cassette was introduced for positive selection of embryonic stem cell clones. (B) Southern blotting experiments verified that out of 200 clones, seven were identified as true homolog recombinants. Detection with a probe for the Neo cassette resulted in a single band, suggesting the absence of extra insertions in the genome. (C) The oligos okk100 (CAGTAATTTTCTTCTTCACTC) and okk101 (ACCTGGTAGGTTCGGATCGGT) were able to detect the wild type and loxP alleles by PCR.

Conflicts of interest

The authors declare no conflicts of interest.

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