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Genetic Identification of Spinal Interneurons that Coordinate Left-Right Locomotor Activity Necessary for Walking Movements

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Summary

The sequential stepping of left and right limbs is a fundamental motor behavior that underlies walking movements. This relatively simple locomotor behavior is generated by the rhythmic activity of motor neurons under the control of spinal neural networks known as central pattern generators (CPGs) that comprise multiple interneuron cell types. Little, however, is known about the identity and contribution of defined interneuronal populations to mammalian locomotor behaviors. We show a discrete subset of commissural spinal interneurons, whose fate is controlled by the activity of the homeobox gene *Dbx1*, has a critical role in controlling the left-right alternation of motor neurons innervating hindlimb muscles. *Dbx1* mutant mice lacking these ventral interneurons exhibit an increased incidence of coburstring between left and right flexor/extensor motor neurons during drug-induced locomotion. Together, these findings identify Dbx1-dependent interneurons as key components of the spinal locomotor circuits that control stepping movements in mammals.

Introduction

The simple repetitive movements that underlie locomotion in nonsessile animals are generated by localized neural networks known as central pattern generators (CPGs) (Arshavsky et al., 1993; Pearson, 1993; Orlovsky et al., 1999). These CPG circuits provide an informative model system for studying how neuronal networks generate simple behaviors. The local interneuron circuits that contribute to the vertebrate locomotor CPG reside in the spinal cord and generate the elemental patterns of motor activity that underlie swimming and walking movements (Orlovsky et al., 1999; Graham-Brown, 1914; Grillner and Zangger, 1979; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997). In primitive vertebrates, such as the lamprey and *Xenopus*, the locomotor CPG that directs swimming movements comprises multiple burst generators organized into "half centers" located on each side of the spinal cord (Arshavsky et al., 1993; Orlovsky et al., 1999). These half centers are mutually inhibitory and connected by commissural interneurons (Arshavsky et al., 1993; Soffe et al., 1984; Cohen and Harris-Warrick, 1984).

Much less is known about the neuronal organization of the locomotor CPG in walking mammals, primarily because of the difficulty in identifying and manipulating its intrinsic interneuronal components. Many of the neurons that contribute to the mammalian locomotor CPG are thought to be located in the ventromedial spinal cord (termed lamina VIII), a region abundant in commissural interneurons that project to the vicinity of contralateral motor neurons (Harrison et al., 1986; Jankowska and Noga, 1990; Butt et al., 2002; Stokke et al., 2002; Birinyi et al., 2003; Butt and Kiehn, 2003). Some lamina VIII commissural interneurons form monosynaptic connections with motor neurons (Harrison et al., 1986; Jankowska and Noga, 1990; Butt et al., 2002; Stokke et al., 2002; Birinyi et al., 2003; Butt and Kiehn, 2003) and are rhythmically active during locomotion (Butt et al., 2002; Butt and Kiehn, 2003), suggesting they have roles in coordinating bilateral flexor and extensor locomotor activity. Consistent with this view, severing the ventral commissure results in a loss in coordinated motor neuron activity in the left and right halves of the spinal cord (Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997; Kato, 1990).

A more precise analysis of the logic by which CPG circuits control locomotor behavior in mammals requires the ability to identify and genetically manipulate the function of individual interneuronal populations and to examine the consequences for locomotion. Recent genetic studies have shown that inactivation of the receptor tyrosine kinase EphA4 results in defects in spinal locomotor function (Kullander et al., 2003); however, identifying the neurons affected by this mutation has been difficult, due to widespread EphA4 expression in the spinal cord (Leighton et al., 2001; Dottori et al., 1998). One potential strategy for selectively manipulating defined sets of CPG interneurons has emerged from recent findings that interneuron subtypes and their progenitors in the developing spinal cord can be distinguished by the restricted expression of homeodomain transcription factors (Jessell, 2000; Lee and Pfaff, 2001; Goulding et al., 2002). These molecularly defined spinal interneuron subtypes appear to be related to functional neuronal subclasses in the developing and mature spinal cord (Goulding et al., 2002; Wenner et al., 2000; Sapir et al., 2004). Moreover, targeted inactivation of these homeobox genes in mice results in the respecification of cell fate, leading to the elimination of specific interneuron populations, while others are produced in greater numbers (Burrill et al., 1997; Ericson et al., 1997; Briscoe et al., 1999; Pierani et al., 2001; Moran-Rivard et al., 2001). Thus, the genetic elimination of defined interneuron subsets through homeobox gene inactivation may permit

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an analysis of their normal contribution to CPG circuitry and locomotor behavior.

Within the developing spinal cord, several distinct classes of commissural inteneurons settle in lamina VIII (Briscoe et al., 1999; Pierani et al., 2001; Moran-Rivard et al., 2001; Gross et al., 2002; Muller et al., 2002; G.M.L. and M.G., unpublished data). Two of these interneuron classes, which we term $V0_V$ and $V0_D$ neurons, differentiate from a discrete progenitor domain, which is marked by expression of the homeodomain transcription factor Dbx1 (Pierani et al., 2001). $V0_V$ interneurons derive from the ventral half of the Dbx1 progenitor domain and transiently express the homeodomain protein Evx1 (Moran-Rivard et al., 2001), whereas $V0_D$ interneurons, which lack Evx1 expression, derive from the dorsal Dbx1 domain (Pierani et al., 2001; G.M.L. and S.G., unpublished data). Both sets of Dbx1-derived interneurons exhibit strikingly similar migratory patterns and initial commissural axonal trajectories (Pierani et al., 2001; Moran-Rivard et al., 2001), suggesting that they are closely related both by provenance and function.

In this study, we have combined genetic and physiological approaches in the mouse to examine the role of Dbx1-derived interneurons in spinal CPG circuitry and locomotion function. Elimination of Evx1 function causes a selective loss of $V0_V$ interneurons, leaving $V0_D$ interneurons intact, whereas eliminating Dbx1 function results in the loss of both $V0_V$ and $V0_D$ interneurons (this study; Pierani et al., 2001; Moran-Rivard et al., 2001). We find that *Evx1* mutant mice exhibit normal locomotor activity, whereas *Dbx1* mutant mice exhibit profound changes in locomotor coordination. *Dbx1* mutant mice exhibit frequent episodes of cocontraction of left and right flexor-extensor motor neurons, whereas phasic ipsilateral L2/L5 flexor-extensor activity persists normally. Our results provide genetic evidence that Dbx1-derived interneurons have a critical role in establishing alternating left-right motor activity during locomotion and thus are critical components of the CPG circuitry that directs locomotor behavior. More generally, our findings illustrate how developmental insights into the transcriptional control of interneuronal subtype specification can be applied to the dissection of local neuronal circuits that regulate locomotor behavior in the mammalian spinal cord.

Results

Dbx1-Dependent Specification of V0 Interneurons

$V0_V$ and $V0_D$ commissural interneurons are generated between E10 and E13.5 and represent the major neuronal progeny of a discrete set of spinal cord progenitors that express the homeodomain protein Dbx1 (Figure 1; Pierani et al., 2001; Moran-Rivard et al., 2001). To monitor V0 interneurons at later developmental stages amenable to physiological analysis, we made use of a *Dbx1*^{lacZ} knockin mouse line in which β-galactosidase (β-gal) expression persists in Dbx1-derived interneurons throughout embryogenesis. In E18.5/P0 *Dbx1*$^{lacZ/+}$ embryos, β-gal$^+$ cells expressing the neuronal marker NeuN were restricted largely to lamina VIII of the spinal cord (Figure 1A, VIII), the location of commissural interneurons that contribute to crossed locomotor pathways (Harrison et al., 1986). A more dorsolateral population of β-gal$^+$ cells was also detected in E18.5/P0 *Dbx1*$^{lacZ/+}$ mice (Figures 1A and 1B). These cells had small nuclei and lacked NeuN expression (Figure 1A, Int.), and many expressed GFAP (data not shown), indicating that they are astrocytes. Thus, as with other ventral spinal progenitor populations (Zhou et al., 2001), Dbx1 progenitors differentiate into both neuronal and glial cell types: initially generating V0 interneurons that settle in and around lamina VIII, and later giving rise to astrocytes.

We have addressed the fate of $V0_V$ and $V0_D$ interneurons in *Dbx1* mutant mice. Previously, we have shown that Evx1$^+$ $V0_V$ neurons are missing in *Dbx1* mutant embryos, a reflection of their early respecification into ipsilaterally projecting inhibitory "V1-like" interneurons (Pierani et al., 2001), which express the homeodomain protein En1 and settle close to motor neurons in lamina IX (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/42/3/375/DC1). In addition, the number of β-gal$^+$/GFAP$^+$ cells in E18.5 *Dbx1* mutants was increased by ~35% (data not shown), suggesting that Dbx1 progenitors destined to form $V0_V$ neurons convert to both V1 interneurons and astrocytes.

The fate of $V0_D$ interneurons in *Dbx1* mutant mice has, however, remained unclear. In *Dbx1* mutants analyzed at E18.5, ~40% of the normal number of β-gal$^+$ neurons persisted in lamina VIII (Figures 1C and 1D), raising the issue of whether these are unchanged $V0_D$ interneurons or a distinct interneuronal population. dI6 commissural neurons, which are derived from progenitors immediately dorsal to the Dbx1 progenitor domain that generates $V0_D$ interneurons, transiently express the homeodomain transcription factor Lbx1 (Gross et al., 2002) and migrate along a similar ventromedial pathway to that of $V0_D$ interneurons (Pierani et al., 2001; Moran-Rivard et al., 2001; Gross et al., 2002). Furthermore, dI6 interneurons, like $V0_D$ interneurons, develop from Pax7$^+$, Dbx2$^+$ progenitors, but differ only in their lack of Dbx1 expression (Pierani et al., 2001; Gross et al., 2002; Muller et al., 2002). We therefore considered the possibility that $V0_D$ interneurons are respecified as dI6 interneurons in the absence of *Dbx1* function. *Dbx1*$^{lacZ/+}$ embryos showed no overlap in the expression of Lbx1 and β-gal (Figures 1E and 1F); however, in *Dbx1*$^{lacZ/lacZ}$ embryos, >80% of β-gal$^+$ neurons in lamina VIII expressed Lbx1 (Figures 1G and 1I), producing a ~25% increase in the total number of Lbx1$^+$ dI6-like interneurons (Figure 1K). These β-gal$^+$ neurons also expressed Pax2 (Figure 1J), consistent with their differentiation as dI6 interneurons (Gross et al., 2002). Many double-labeled β-gal$^+$/Lbx1$^+$ neurons were detected in the dorsal Dbx1$^+$ subventricular zone of *Dbx1*$^{lacZ/lacZ}$ embryos (Figure 1H), demonstrating that prospective "$V0_D$" neurons acquire a dI6 molecular character soon after their generation.

We next examined the selectivity of ventral interneuron loss in *Dbx1* mutants. Sim1$^+$ V3 interneurons, BarH1$^+$ dI1 interneurons, and Brn3a$^+$/Lmx1b$^+$ dI5 interneurons, which are all commissural interneuron cell types, were generated in normal numbers and occupied appropriate settling positions in the spinal cord of *Dbx1* mutants (Supplemental Figure S2 at http://www.neuron.

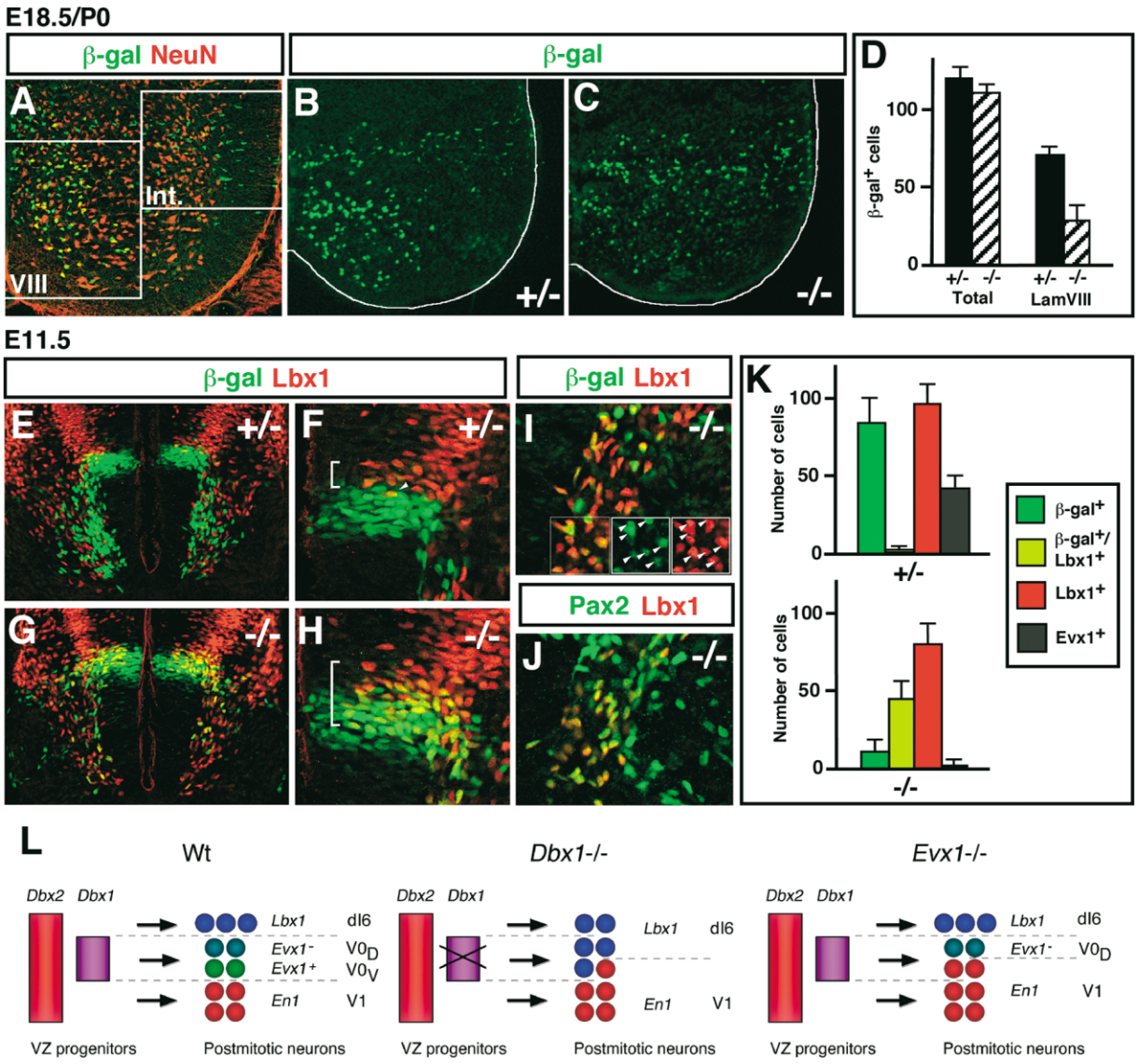

Figure 1. Loss of V0 Interneurons in *Dbx1* Mutant Mice

(A) Lumbar P0 *Dbx1*$^{lacZ/+}$ spinal cord (sc) stained with antibodies to β-galactosidase (β-gal) and the neuronal marker NeuN. Most of the Dbx1-derived cells in lamina VIII (VIII) express NeuN, whereas those in the intermediate sc (Int.) have smaller nuclei and are negative for NeuN. N.B.: the ratio of β-gal$^+$ neurons to total NeuN$^+$ ventral cells is unchanged from E13 (14%) to E18.5 (15%), indicating β-gal is an excellent lineage marker for these neurons.

(B and C) β-gal expression in the lumbar sc of an E18.5 *Dbx1*$^{lacZ/+}$ (B) and a *Dbx1*$^{lacZ/lacZ}$ (C) embryo. Note the decrease in lamina VIII β-gal$^+$ cells and the concomitant increase in β-gal cells in the intermediate region of the mutant sc in (C).

(D) Comparison of Dbx1-derived (β-gal) cell numbers in the E18.5 lumbar sc of *Dbx1*$^{lacZ/+}$ heterozygote (solid) and *Dbx1*$^{lacZ/lacZ}$ mutant (hatched) embryos (left). There is a marked reduction in the number of Dbx1-derived cells in lamina VIII (right).

(E–L) Respecification of V0$_D$ interneurons in the *Dbx1* mutant sc.

(E and F) Cross-sections through an E11.5 *Dbx1*$^{lacZ/+}$ sc showing V0 (β-gal$^+$, green) and dI6 (Lbx1$^+$, red) neurons form distinct populations that migrate ventrally toward the floor plate. (F) Double labeled cells (arrowhead) are only rarely observed in the spinal cord of *Dbx1*$^{lacZ/+}$ heterozygotes.

(G–J) Cross-sections through an E11.5 *Dbx1*$^{lacZ/lacZ}$ sc showing V0$_D$ neurons that arise in the dorsal p0 domain express Lbx1. Ventrally migrating Dbx1-derived neurons express Lbx1 (H and I) and Pax2 (J).

(K) β-gal, Lbx1, and Evx1 cell numbers in lamina VIII.

(L) Summary of changes in V0 cell fate in the *Dbx1* and *Evx1* mutant spinal cord.

org/cgi/content/full/42/3/375/DC1). Moreover, TAG1$^+$ axons were detected in the ventral commissure of wild-type and *Dbx1*$^{lacZ/lacZ}$ embryos (Supplemental Figure S2), indicating that dorsal commissural neurons still project axons contralaterally in the absence of Dbx1 function. Together with previous studies (Pierani et al., 2001), these data provide evidence for the selective loss of V0 interneurons in the *Dbx1* mutant spinal cord. These

findings also indicate that prospective $V0_V$ and $V0_D$ interneuron progenitors generate V1 and dI6 interneurons, respectively.

V0 Commissural Interneurons Form Inhibitory Connections with Motor Neurons

Both sets of V0 interneurons extend axons contralaterally (Pierani et al., 2001; Moran-Rivard et al., 2001); however, their postsynaptic targets have not been defined. To assess whether V0 interneurons contact motor neurons, we examined whether introduction of a transynaptic tracer into motor neurons resulted in the rapid labeling of V0 interneurons. A GFP-expressing Bartha strain of Pseudorabies Virus (PRV152), which is known to be transported transynaptically (Smith et al., 2000; Kerman et al., 2003), was injected unilaterally into various hindlimb muscles of P1 *Dbx1*$^{lacZ/+}$ mice. Following injections into the gastrocnemius muscle, ipsilateral motor neurons in spinal segments L3 and L4, the location of the gastrocnemius motor pool, were selectively labeled with GFP at 24 hr (Figure 2B). At 36 hr, GFP-labeled, β-gal$^+$ V0 interneurons were present in the contralateral, but not ipsilateral, ventral lumbar spinal cord (Figure 2E, data not shown), consistent with their identity as commissural interneurons. By 48 hr, increased numbers of labeled V0 interneurons were detected contralaterally, along with other sets of β-gal$^-$ ventral commissural interneurons (Figures 2C and 2F). A number of β-gal$^-$ ipsilateral interneurons were also labeled with GFP, demonstrating widespread transynaptic labeling of premotor neuron cell types by PRV152 (data not shown).

The short latency (36 hr) between virally transduced GFP expression in motor neurons and the appearance of GFP in V0 interneurons (Figure 2E) argues that many V0 interneurons form direct commissural connections with contralateral motor neurons. Injection of PRV152 virus into either the extensor-related biceps femoris (Figure 2D) or the flexor-related semitendinosus muscles (data not shown) also resulted in the retrograde labeling of contralateral β-gal$^+$ V0 interneurons, indicating that V0 interneurons innervate multiple motor neuron pools in the mouse hindlimb. We observed that ~70% of the contralateral lamina VIII neurons labeled with GFP after muscle injection of PRV152 lacked β-gal expression (see Figures 2D, arrow, 2E, and 2F), demonstrating that V0 neurons are not the only lamina VIII neurons that form commissural connections with motor neurons.

A role for inhibitory commissural interneurons in left-right alternation during swimming and walking movements has been noted in a variety of vertebrate species (Arshavsky et al., 1993; Cohen and Harris-Warrick, 1984; Butt and Kiehn, 2003; Buchanan, 1982; Soffe and Roberts, 1982). However, the origin and identity of these inhibitory commissural interneurons has not been determined. To assess whether V0 interneurons constitute an inhibitory class of lamina VIII neurons, we analyzed the distribution of the vesicular inhibitory amino acid transporter (VIAAT) that delineates both GABAergic and glycinergic neurons (Sagne et al., 1997). It is presently not known whether these inhibitory V0 interneurons utilize GABA, glycine, or both as neurotransmitters. In E18.5 *Dbx1*$^{lacZ/+}$ mice, ~70% of β-gal$^+$ V0 neurons in lamina VIII expressed VIAAT (Figure 3A). However, the presence of β-gal$^+$ V0 neurons in lamina VIII that lacked VIAAT expression raised the possibility that not all V0 neurons express inhibitory transmitters. In support of this, ~30% of β-gal$^+$ neurons in lamina VIII expressed VGlut2 (Figure 3B), a marker of glutamatergic interneurons (Bai et al., 2001; Takamori et al., 2001). These findings nevertheless suggest that the majority of V0 interneurons release inhibitory amino acid neurotransmitters.

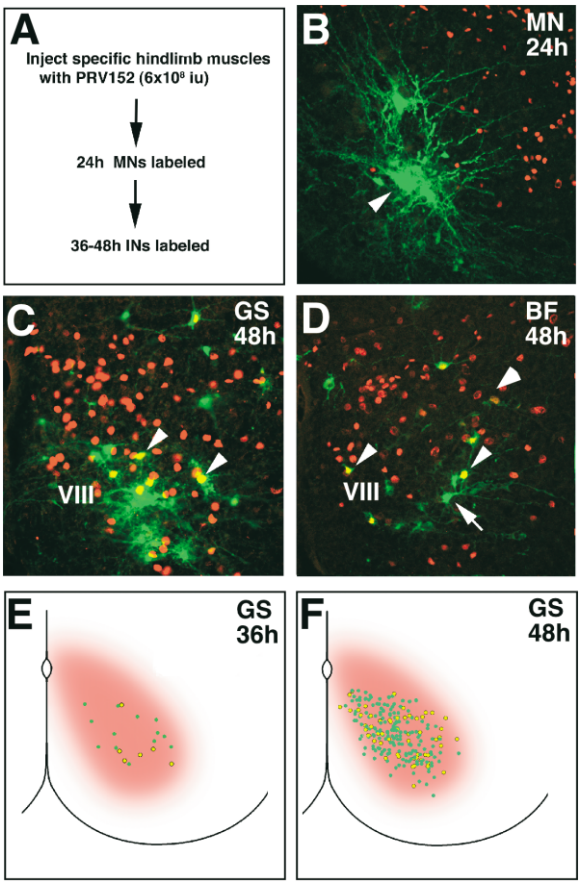

Figure 2. V0 Interneurons Synapse with Motor Neurons

(A) Summary of the PRV labeling protocol.

(B) PRV152-labeled motor neurons (MN, green) 24 hr after injecting the gastrocnemius.

(C and D) PRV-labeled V0 interneurons (yellow, arrowheads) 48 hr after injecting the gastrocnemius (GS) and biceps femoris (BF) muscles.

(E and F) Schematic showing lamina VIII commissural neurons in L3 that are (yellow = V0, green = non-V0) transynaptically labeled from GS motor neurons at 36 and 48 hr.

V0 Interneurons Are Activated during Fictive Locomotion

We next asked whether β-gal$^+$ V0 neurons within lamina VIII are activated during locomotor-like tasks and are therefore likely to contribute to the spinal circuits controlling locomotion. We reasoned that if V0 interneurons are integral components of the spinal CPG, we might observe changes in c-*fos* expression in these neurons

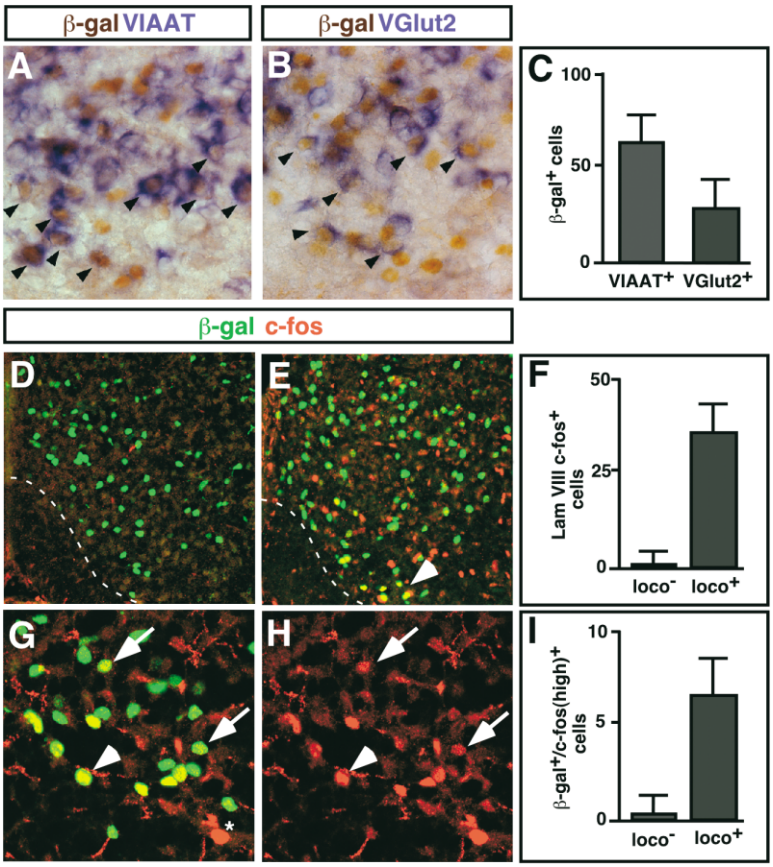

Figure 3. Locomotor-Dependent Induction of c-*fos* in V0 Interneurons

(A) Cross-section though E18.5 *Dbx1*$^{lacZ/+}$ spinal cord showing VIAAT expression (purple) in many β-gal$^+$ (brown) V0 interneurons (arrowheads mark double labeled cells).

(B) Parallel section showing VGlut2 expression (purple) in a subset of V0 interneurons (brown).

(C) Number of β-gal$^+$/VIAAT$^+$ and β-gal$^+$/VGlut2$^+$ cells per 20 μm hemichord sections from E18.5 *Dbx1*$^{lacZ/+}$ spinal cords (n = 15 sections).

(D and E) c-*fos* expression (red) in lamina VIII of a control E18.5 *Dbx1*$^{lacZ/+}$ spinal cord before (D) and after (E) inducing locomotion.

(F) Number of c-*fos*$^+$ neurons in lamina VIII before (loco$^-$) or after (loco$^+$) locomotion. Cell counts are for 20 μm hemichord sections, n = 5 sections.

(G and H) High-level expression of c-*fos* (red) in V0 interneurons (green, double labeled cells are yellow, arrowhead); N.B.: the c-*fos* protein is located in the nuclei of these neurons (H, arrowhead). Asterisk in (G) indicates c-fos (high) non-V0 interneuron. Low-level c-fos expression is seen in many V0 interneurons (arrows).

(I) Number of β-gal$^+$/c-*fos* (high) neurons in lamina VIII per 20 μm hemichord section (n = 12 sections).

following a locomotor-like task (Barajon et al., 1992; Jasmin et al., 1994; Carr et al., 1995). We examined c-*fos* expression in isolated spinal cord preparations before and after inducing fictive locomotion with NMDA and 5-HT. In control "nonlocomoting" spinal cords (n = 3), few, if any, V0 interneurons expressed c-*fos* (Figures 3D and 3F). In contrast, induction of locomotor-like motor neuron activity in isolated *Dbx1*$^{lacZ/+}$ spinal cords (n = 4) resulted in a marked increase in c-*fos* expression in V0 neurons (Figures 3E–3I). Low levels of c-*fos* were observed throughout lamina VIII, in scattered β-gal$^+$ V0 interneurons (Figures 3G and 3H, arrows), but a subset of V0 interneurons in the ventral part of lamina VIII close to the white matter typically exhibited a high level of c-*fos* induction (Figures 3G and 3H, arrowheads). The activity-induced upregulation of c-*fos* protein expression in V0 interneurons supports the idea that these neurons are among those recruited during locomotor behaviors.

Left-Right Alternation in Motor Output Is Unaffected in *Evx1* Mutants

Since commissural connections in the ventral spinal cord are necessary for left-right alternation (Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997; Kato, 1990), we examined the impact of the loss of V0 interneurons on locomotor behavior in an in vitro spinal cord preparation that exhibits NMDA-induced locomotor-like activity (Figure 4A; Kudo and Yamada, 1987; Smith and Feldman, 1987). We first assessed whether V0$_V$ interneurons have an essential role in coordinating left-right alternation by examining fictive locomotor activity in the *Evx1* mutant spinal cord. *Evx1* mutant mice exhibit a 65%–70% reduction in the number of V0$_V$ interneurons, and the residual V0$_V$-like interneurons fail to project axons intersegmentally on the contralateral side of the spinal cord (Moran-Rivard et al., 2001). In both E18.5/P0 wild-type (Figure 4B) and *Evx1*$^{-/+}$ (data not shown) mice, application of NMDA (5 μM) and 5-HT (5–15 μM) induced rhythmic "walking" activity in the isolated mouse spinal cord. This activity was characterized by strictly alternating left-right flexor- or left-right extensor-related ENG motor activity, as recorded from the ipsilateral L2 and L5 ventral roots, respectively (Kjaerulff and Kiehn, 1996; Smith and Feldman, 1987). This pattern shares many similarities with walking movements in adult rodents (Gruner et al., 1980).

E18.5/P0 *Evx1* mutant spinal cords (n = 8) also exhibited an invariant alternating pattern of motor activity between the left L2 (lL2) and right L2 (rL2) flexor-related nerves (Figure 4C). Analysis of 940 episodes of L2 motor bursting did not reveal any episodes of cobursting between the left and right L2 ventral roots (see Table 1). Nor did we observe cobursting of left and right L5 extensor-related motor neurons (data not shown). *Evx1* mutant spinal cords also showed appropriate alternation of flexor/extensor-related motor activity at hindlimb levels

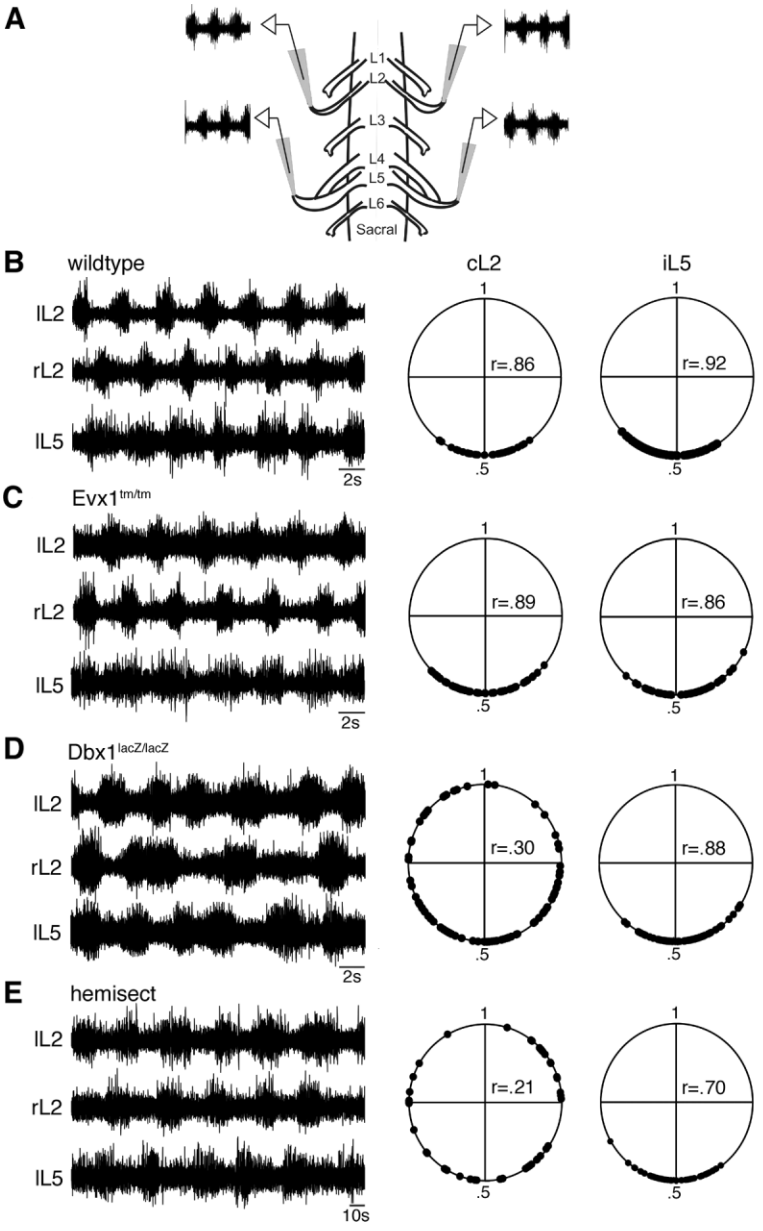

Figure 4. *Dbx1* Mutant Mice Display Synchronous Activity between Right and Left L2 Ventral Roots during Locomotor-like Activity

(A) Schematic of recording setup with suction electrodes recording ENG activity from left and right L2 (lL2, rL2) and left and right L5 (lL5, rL5) ventral roots. Application of NMDA (5 μM) and 5-HT (10 μM) results in a locomotor-like pattern of activity.

(B–E) ENG recordings from lL2, rL2, and rL5 ventral roots (left). Appropriate left-right alternation is observed in wild-type (B) and *Evx1*$^{tm/tm}$ (C) spinal cords. *Dbx1*$^{lacZ/lacZ}$ mutant (D) and hemisected (E, T8-caudal extent of spinal cord) spinal cords exhibit periods of synchronous activity in the lL2 and rL2 ventral roots (middle). In all instances, there is appropriate alternation between lL5 and both L2 ventral roots (right). All circular plots show coupling of rL2 (cL2) and lL5 (iL5) bursts with respect to lL2 bursts over a 5 min period of activity. Points located near 0.5 represent alternation, while those located close to 1 represent synchronicity. r values are shown for each polar plot.

(Figure 4C, cf. lL2 and lL5). Furthermore, adult *Evx1* mutant animals did not exhibit gait changes in locomotor behavioral tests (G.M.L. and S.G., unpublished data; Moran-Rivard et al., 2001). Thus, neither the selective depletion of $V0_V$ interneurons nor the concommitant overproduction of V1 neurons is sufficient to impair motor coordination.

Dbx1 Mutant Mice Exhibit Marked Changes in Left-Right Motor Coordination

We next asked whether the elimination of $V0_D$ as well as $V0_V$ interneurons, a situation achieved in *Dbx1* mutant mice, results in defects in locomotor behavior. Once again we used the in vitro spinal cord preparation, since *Dbx1* mutant mice die at birth (Pierani et al., 2001). Strik-

Table 1. Averaged Values for Step Cycle, Burst Period, and Cobursting in the In Vitro Spinal Cord Locomotor Preparation

	Step Period (s)	Burst Period (s)	In Phase Bursting (%)	r Value (± SD)
Wild-type (n = 8)	3.41 ± .31	1.87 ± .47	0	0.88 ± .03
Evx1$^{tm/tm}$ (n = 8)	3.28 ± .97	1.69 ± .24	0	0.88 ± .05
Dbx1$^{lacZ/+}$ (n = 7)	3.65 ± .46	1.66 ± .66	0	0.85 ± .05
Dbx1$^{lacZ/lacZ}$ (n = 12)	3.78 ± .58	1.84 ± .69	22	0.52 ± .15
Dbx1$^{lacZ/lacZ}$ + sarcosine (n = 9)	3.91 ± .77	1.90 ± .88	10	0.71 ± .10
Dbx1$^{lacZ/lacZ}$ + nip. acid (n = 5)	5.19 ± .47	1.94 ± .18	12	0.56 ± .07

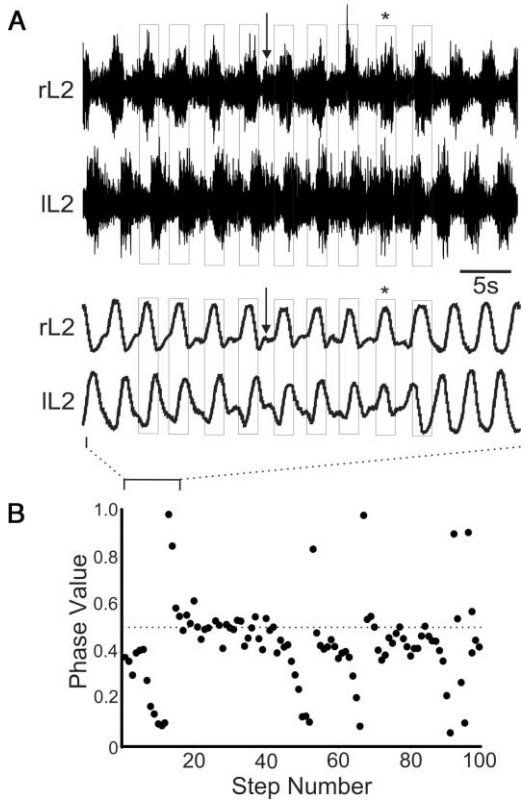

Figure 5. Synchronous Bursting of Contralateral Ventral Roots Often Has a Gradual Onset

(A) Top, ENG traces showing motor neuron activity in the left and right L2 ventral roots during locomotor-like activity in a *Dbx1*$^{lacZ/lacZ}$ mouse. Bottom, rectified integrated ventral root recordings of the traces shown at top.

(B) Phase values of rL2 (with respect to lL2) for 100 consecutive bursts. In many instances, the onset of cobursting between contralateral ventral roots was gradual, until complete synchronicity occurred (asterisk in A). During periods of synchronous activity between contralateral L2 ventral roots, small minibursts (indicated by an arrow in A) would often occur during the inhibition phase of the step cycle. These minibursts were absent during periods of appropriate left-right alternation. Boxes indicate periods of significant miniburst activity.

ingly, isolated spinal cords from E18.5 *Dbx1*$^{lacZ/lacZ}$ mice exhibited frequent episodes of overlap in the bursting of left and right L2 flexor-related motor neurons during periods of drug-induced locomotion (Figure 4D, left panel, arrow). Similar episodes of L5 ventral root cobursting also occurred (data not shown). Episodes of synchronous bursting in *Dbx1* mutants were interspersed with periods of normal alternation, suggesting a drift in the phasing of motor activity between the left and right halves of the spinal cord (Figure 5). Using polar plots to map the onset of the step cycle in rL2 with respect to lL2 (Kjaerulff and Kiehn, 1996; Butt et al., 2002), we typically observed phasing centered around 0.5 of the step cycle and r values close to 1 in wild-type and heterozygous *Dbx1*$^{lacZ/+}$ spinal cord (Figure 4B, middle panel), indicating normal left-right alternation. In contrast, the onset of the contralateral step cycle in *Dbx1* mutant mice was highly variable (mean r value = 0.52), with many (22%) rL2 bursts exhibiting phase shifts of $<.25$ or $>.75$ with respect to lL2, indicative of synchronous activation of the lL2 and rL2 ventral roots (Figure 4D, middle panel). The loss of left-right alternation observed in *Dbx1* mutants resembles in part that reported after partial hemisection of the lower thoracic/lumbar spinal cord (Figure 4E; Cowley and Schmidt, 1997; Soffe et al., 1984).

Despite the loss of left-right motor coordination, alternating L2 flexor-related and L5 extensor-related motor activity was maintained in the *Dbx1* mutant spinal cord (Figure 4D, right panel, r = 0.89). Thus, V0 interneurons are not necessary for flexor-extensor alternation. No other overt differences in rhythmic motor activity were evident in the *Dbx1* mutant spinal cord. The step cycle periods in wild-type and *Dbx1* mutant spinal cord did not differ significantly (Table 1; wt, 3.41 s versus *Dbx1*$^{-/-}$, 3.82 s), nor did we observe any significant lengthening in the burst phase of the step cycle (Table 1; wt, 1.87 s versus *Dbx1*$^{-/-}$, 1.86 s) that might account for the overlap in left-right L2 motor activity. In contrast, complete hemisection of the spinal cord resulted in a substantial lengthening of the step cycle (12.81 s, hemisected versus 3.41 s, wt) (Figure 4E; Kjaerulff and Kiehn, 1996). The Dbx1-dependent elimination of V0 interneurons therefore produces a more selective disruption in locomotor coordination than does ventral commissure transection.

The impaired motor behavior of *Dbx1* mutant mice was also accompanied by frequent episodes of enhanced ventral root activity during the inhibition phase of the step cycle (Figure 5). These "minibursts" typically accompanied periods in which there was a progressive shift in the relative phasing of left-right motor neuron activity. The rectified trace in Figure 5 illustrates a series of minibursts that precede each major burst in rL2 during the inhibition phase of the step cycle (see arrow in Figure 5A for an example). This episodic miniburst activity during the inhibition phase was typically accompanied by a slow change in the relative phasing of the left and right spinal cord halves (Figure 5A, boxes) and led eventually to the synchronous firing of left and right L2 ventral roots (Figure 5A, asterisk). Strikingly, such minibursts in the inhibition phase of the step cycle were not evident during periods of normal alternation, indicating a close correlation between supernumerary miniburst activity and the loss of left-right alternation. During periods of stable drug-induced locomotion, *Dbx1* mutants typically underwent multiple episodes of phase shifting and synchronous left-right motor activity (Figure 5B).

Enhanced Inhibitory Transmission Can Restore Left-Right Alternation in *Dbx1* Mutants

Most V0 interneurons missing in *Dbx1* mutants are inhibitory commissural interneurons (Figure 3A), raising the possibility that V0 interneurons normally function by inhibiting contralateral motor neurons during the inhibition phase of the step cycle. We reasoned, therefore, that enhancing inhibitory transmission might prevent the abnormal activation of motor neurons during the inhibition phase of the cycle, thereby restoring the normal left-right alternation of motor outputs. We tested this idea by applying the transmitter reuptake inhibitors sarcosine (100 μM) or nipecotic acid (120 μM) to potentiate gly-

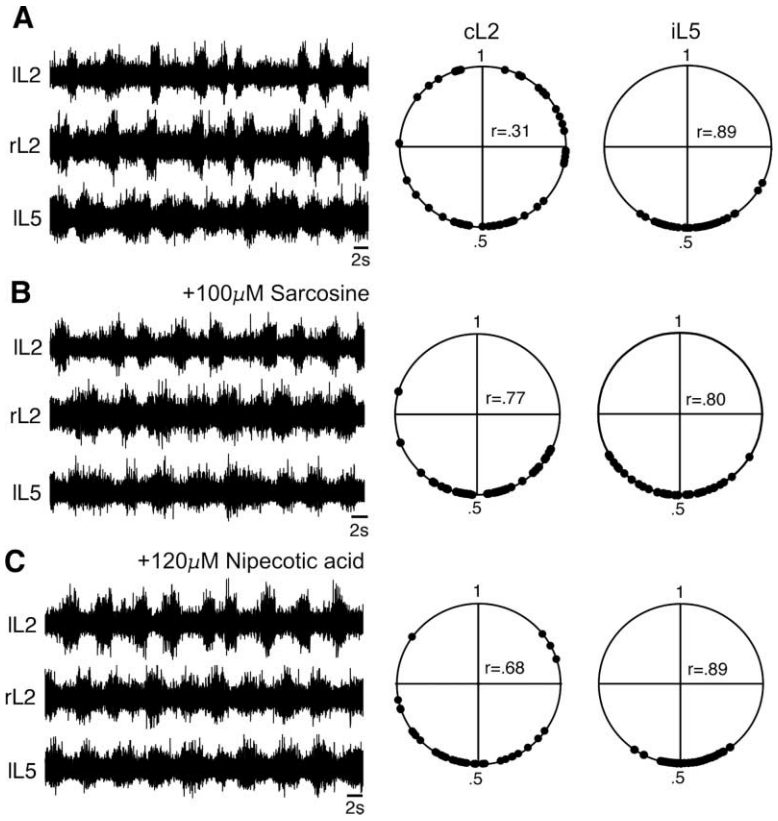

Figure 6. Effect of Inhibitory Neurotransmitter Agonists on Locomotor-like Activity in the *Dbx1* Mutant Spinal Cord

Left: Locomotor-like activity recorded from lL2, rL2, lL5 ventral roots following drug application.

(A) Typical episode of locomotor like activity in a *Dbx1* mutant cord showing occasional episodes of synchronous activity.

(B) ENG activity in the same cord preparation after addition of 100 μM sarcosine.

(C) Synchronous bursting is reduced upon addition of 120 μM nipecotic acid.

Middle and right: Polar plots (5 min duration) indicating the phasing of bursts in rL2 and lL5 compared to lL2. r values are shown for each polar plot.

cinergic and GABAergic transmission, respectively. In the *Dbx1* mutant spinal cord, application of either sarcosine or nipecotic acid significantly lowered the incidence of left-right cobursting during NMDA- and 5-HT-elicited locomotion (Figure 6, Table 1) and at the same time reduced the number of minibursts (data not shown). These findings are consistent with the idea that the loss of left-right motor coordination observed in *Dbx1* mutant spinal cord results from an impairment in inhibitory inputs between commissural neurons and motor neurons.

Discussion

The mammalian central nervous system exhibits a modular organization, in which localized networks of interneurons play critical roles in controlling neural function. Dissecting the contribution of individual classes of interneurons to defined behaviors has, however, proved problematic. Using mouse genetics, we have identified a discrete population of spinal CPG interneurons necessary for walking movements. Mice lacking the homeodomain transcription factor Dbx1 show a selective depletion of V0 commissural interneurons and exhibit a specific locomotor phenotype characterized by increased cobursting of left and right flexor or extensor motor neuron populations. These findings establish a role for Dbx1 in specifying the identity of interneurons critical to the organization of spinal locomotor circuits that control stepping movements. Our findings also indicate how the genetic manipulation of transcription factors can be used to assess the behavioral function of specific classes of interneurons embedded within neural networks. Since transcription factors serve as determinants of interneuronal identity throughout the CNS, this strategy may permit a more effective analysis of the local circuits that control diverse mammalian behaviors.

Assignment of Locomotor Defects to the Loss of V0 Interneurons

In mice lacking *Dbx1*, many of the neural progenitor cells that normally give rise to V0 neurons instead generate V1 and dI6 interneurons. The respecification of ventral interneurons evident in *Dbx1* mutants therefore raises the issue of whether the aberrant *Dbx1* mutant locomotor phenotype is caused by the loss of V0 interneurons or by the generation of supernumerary dI6 and V1 interneurons. Several lines of evidence support the view that the loss of V0 interneurons is the primary cause of the locomotor phenotype evident in *Dbx1* mutants. While some prospective $V0_V$ interneurons give rise to $En1^+$ V1 interneurons in the absence of *Dbx1* function, the net increase in V1 interneuron number in older animals is small ($<$10% at E18.5; Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/42/3/375/DC1). More tellingly, a similar switch from $V0_V$ to V1 interneuronal fate occurs in *Evx1* mutant mice, in the absence of any overt locomotor defects (Figure 4; Moran-Rivard et al., 2001). In addition, V1 interneurons are absent in *Pax6* mutants (Burrill et al., 1997; Ericson et al., 1997), yet the left-right alternation of locomotor output is unaffected (S.G., S. Butt, O. Kiehn, and M.G., unpublished data). Consequently, the minor change in V1 interneuron generation in *Dbx1* mutants is unlikely to cause the degradation of left-right locomotor phasing.

In principle, the ~25% enhancement in the generation of dI6 neurons in *Dbx1* mutant mice might contribute to the observed locomotor defects. However, dI6 commissural interneurons, like the missing V0 interneurons, are predominantly inhibitory (G.M.L. and S.G., unpublished data), and accordingly, >85% of the residual β-gal$^+$ "dI6" interneurons in the *Dbx1* mutant spinal cord express VIAAT, but not VGlut2 (Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/42/3/375/DC1). These findings are not easily reconciled with the fact that the *Dbx1* mutant phenotype appears to reflect a loss of inhibitory inputs to contralateral motor neurons. Moreover, if the overproduction of dI6 interneurons was the underlying cause of the *Dbx1* locomotor phenotype, the addition of drugs that enhance inhibitory transmission would be expected to increase, rather than decrease, the penetrance of the mutant locomotor phenotype. Indeed, the modest increase in dI6 interneurons observed in *Dbx1* mutants is more likely to underlie the incomplete disruption of left-right alternation than to represent the underlying cause of the locomotor defects. Thus, the locomotor defects in *Dbx1* mutants are most easily explained by the loss of V0 interneurons, rather than by the overproduction of V1 or dI6 interneurons.

The respective contributions of individual V0 interneuronal subpopulations to the left-right alternation of motor output remains to be determined. $V0_D$ interneurons could have a more critical role than $V0_V$ interneurons in the inhibition of contralateral motor neurons, thereby accounting for the detection of a locomotor phenotype in *Dbx1* but not *Evx1* mutants. Alternatively, the two sets of V0 neurons might have similar and additive roles in mediating contralateral motor neuron inhibition, in which case the elimination of both neuronal populations might be necessary to produce a detectable locomotor phenotype. Nevertheless, the persistence of dI6 inhibitory commissural interneurons in *Dbx1* mutants does not compensate completely for the loss of V0 neurons, raising the possibility of functional selectivity in the actions of molecularly distinct populations of commissural inhibitory interneurons.

A Selective Locomotor Defect in the Absence of Inputs from V0 Interneurons

Our results indicate that V0 interneurons are key components of the commissural pathways that maintain proper alternation between the limbs during locomotion. Two observations, however, suggest that additional, V0-independent, commissural pathways are involved in controlling left-right motor alternation. First, viral tracing studies show that V0 interneurons represent only a minority of the lamina VIII commissural interneurons that synapse onto motor neurons. Second, surgical transection of the ventral commissure results in the complete uncoupling of alternating motor activity between the left and right halves of the spinal cord (this study; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997). Importantly, transection of the ventral commissure also causes a marked slowing in locomotor rhythm (Figure 4; Kjaerulff and Kiehn, 1996), suggesting that commissural neurons may play an additional role in determining the duration of the step cycle. There is no slowing of rhythmic motor activity in the *Dbx1* mutant spinal cord (Table 1), suggesting that V0 interneurons are involved selectively in the coordination of left-right motor alternation.

The view that the locomotor defects detected in *Dbx1* mutants result from the loss of inhibitory commissural inputs to contralateral motor neurons is supported by the finding that inhibitors of glycine and GABA reuptake reduce the incidence of coboursting in the *Dbx1* mutant spinal cord (Figure 6, Table 1). One possibility is that these agents restore normal motor function by increasing the inhibitory drive from dI6 interneurons, which are still present in the *Dbx1* mutant spinal cord, thereby permitting these neurons to compensate for the loss of V0 interneurons. This interpretation is consistent with surgical studies, in which a decrease in excitatory locomotor drive restores the reduction in left-right coupling elicited by partial transection of the ventral commissure (Kjaerulff and Kiehn, 1996). Our genetic analysis, together with lesion and pharmacological studies, therefore suggest a model in which V0 interneurons represent an important class of commissural interneurons that contribute to the net threshold of contralateral inhibitory input needed to maintain appropriate left-right motor alternation.

The finding that a minority of V0 interneurons release excitatory transmitters raises the additional question of how this subset of V0 interneurons regulates contralateral motor activity. In the neonate rat spinal cord, some lamina VIII excitatory commissural neurons form connections with contralaterally located inhibitory interneurons that, in turn, innervate motor neurons (Butt and Kiehn, 2003). This raises the possibility that the excitatory subset of V0 interneurons may inhibit motor output through a disynaptic pathway.

Molecular Genetic Dissection of Spinal CPG Interneuronal Circuitry

Recent studies in neonate rats have identified distinct populations of commissural interneurons that are rhythmically active during locomotion (Butt et al., 2002; Butt and Kiehn, 2003). The relationship of these physiologically defined neurons to V0 interneurons is not known. Nevertheless, our observation that the elimination of a discrete set of inhibitory commissural interneurons produces a selective motor behavioral phenotype provides strong evidence that homeodomain transcription factors such as Dbx1 and Evx1 specify the identity of functional interneuronal types that contribute to spinal locomotor circuits.

Genetic perturbations of cell surface receptor signaling between spinal cord cells can also influence locomotor function (Kullander et al., 2003). In particular, defective EphA4 kinase signaling in the mouse spinal cord results in the synchronization of left-right hindlimb motor activity (Kullander et al., 2003; Dottori et al., 1998). The expression of EphA4 in multiple spinal neuron cell types (Leighton et al., 2001; Dottori et al., 1998) has, however, made it difficult to ascribe this locomotor phenotype to an identifiable interneuronal subtype. Moreover, the *EphA4* mutant locomotor defect most likely reflects a gain-of-function phenotype, as it has been proposed that ipsilaterally projecting excitatory interneurons form ectopic connections with contralateral motor neurons

in the absence of EphA4 function (Kullander et al., 2003). Thus, it remains unclear whether neurons misrouted in *EphA4* mutant mice normally participate in the neural circuits that control left-right alternation.

The emergence of defined locomotor deficits in *Dbx1* and *EphA4* mutant mice nevertheless demonstrates that the genetic manipulation of spinal interneuron fate and connectivity can provide useful insights into spinal CPG circuitry and its link to locomotor behavior. The remarkable selectivity of transcription factor expression by subsets of spinal cord interneurons (Jessell, 2000; Lee and Pfaff, 2001; Goulding et al., 2002) offers a particularly powerful and systematic way of dissecting local interneuronal function (Kiehn and Kullander, 2004), either through the strategy of cell fate switching illustrated in this study or through the neuronal subtype-restricted expression of toxins and membrane proteins that kill neurons or silence their activity (Lee et al., 2000; Baines et al., 2001). Our findings also highlight the potential for using transcription factors as genetic tools to functionally analyze the neuronal circuits controling other important mammalian behaviors such as respiration or visual perception. Finally, the identification of interneurons with critical roles in locomotion should aid in the targeted design of more effective therapies for the recovery of coordinated motor function after spinal cord injury.

Experimental Procedures

Animals

The generation of *Dbx1*^{lacZ} and *Evx1*^{taumyc} mutant mice has been described previously (Pierani et al., 2001; Moran-Rivard et al., 2001). Embryos were obtained from timed matings with the morning of the vaginal plug designated as E0.5. Genotyping of mice was performed by PCR as previously described (Pierani et al., 2001; Moran-Rivard et al., 2001).

In Situ Hybridization and Immunohistochemistry

In situ hybridization was performed as described previously (Gross et al., 2002). The following in situ probes were used: mouse *VIAAT*, *VGlut2*, *Sim1*, and *BarhL1* (see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/42/3/375/DC1 for references). For antibody in situ double staining, the proteinase K treatment step was omitted and sections were incubated with an antibody to β-gal (Cappel) after developing the DIG in situ reaction.

Immunostaining on frozen spinal sections was performed as previously described (Gross et al., 2002; Moran-Rivard et al., 2001). 20 μm serial sections were cut and incubated with primary antibodies. Primary antibodies were detected using species-specific secondary antibodies conjugated with Cy3 or Cy2 (Jackson Laboratories). Images were captured using a Zeiss LSM510 confocal microscope and assembled using Photoshop.

c-*fos* Analysis

Spinal cords isolated from P0 *Dbx1*$^{lacZ/+}$ heterozygous embryos were placed in a recording chamber and perfused with Ringers (see below). Locomotion (monitored with extracellular suction electrodes) was induced by the addition of 5 μm NMDA and 5–15 μm serotonin. After 1 hr of stable activity, spinal cords were perfused with ice-cold 4% paraformaldehyde-PBS. Cryostat sections taken from the lumbar cord were stained with antibodies to c-*fos* (Oncogene) and β-gal (Gross et al., 2002). In control experiments, NMDA and 5-HT were omitted.

PRV Tracing

Injections of PRV into hindlimb muscles were performed according to Kerman et al. (2003). Typically 1–2 μl of Bartha PRV152 viral stock (2×10^8 infectious units per μl) was pressure injected sterotaxically into a single identified hindlimb muscle at P1 using glass micropipettes. Animals were sacrificed 24–48 hr after injecting them. Spinal cords were dissected out in ice-cold PBS before fixation in 4% paraformaldehyde-PBS at 4°C. Sections from the lumbar spinal cord were stained with antibodies to GFP and β-gal to visualize *trans*-synaptically labeled V0 interneurons.

Electrophysiology

Electrophysiological experiments were performed on embryonic (E18.5) or early postnatal (P0) mice. Animals were anesthetized with halothane, decapitated, and eviscerated. Spinal cords were dissected out in ice-cold Ringers solution (Kjaerulff and Kiehn, 1996) and pinned, ventral side up, in a recording chamber constantly perfused with oxygenated Ringer's solution composed of 111 mM NaCl, 3.08 mM KCl, 11 mM glucose, 25 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 1.25 mM $MgSO_4$, 2.52 mM $CaCl_2$. All recordings were made at room temperature (20°C).

ENG Recordings

The second and fifth lumbar ventral roots on the right and left (i.e., rL2, lL2, rL5, lL5) were placed in suction electrodes. Electroneurogram (ENG) recordings were amplified, bandpass filtered (100 Hz–1 kHz), digitized, collected, and stored on a PC using the Axoscope software (Axon Instruments). Rhythmic locomotor activity was induced in the ventral roots by adding *N*-methyl-D-aspartic acid (NMDA, 5 μM) and 5-hydroxytryptamine (5-HT, 5–15 μM) to the perfusing Ringer's solution. The effects of sarcosine (100–150 μM) and nipecotic acid (100–150 μM) were investigated by adding these drugs to the NMDA/5-HT Ringer's solution.

Analysis of Locomotor Activity

Circular statistics (Zar, 1974) were used to determine the coupling strength between opposing L2 and L5 ventral roots. Left L2 (lL2) bursts occurring over a continuous 5 min interval (1/3 of the total recording time) were selected, and their phase values were calculated in reference to either the onsets of each rL2 or lL5 burst. Phase values were determined by dividing the latency between the onset of the first lL2 burst and the following burst in rL2 (or lL5) by the step cycle period (time between the reference lL2 burst and the next lL2 burst). Locomotor steps in which the lL2 and rL2 roots were completely out of phase (i.e., appropriate left-right alternation) had phase values of ~0.5. Those completely in phase (cobursting) had phase values of ~1. The r values are a measure of the concentration of phase values around the mean value for alternation (0.5). An r value of 1 indicates all the phase values are 0.5, whereas an r value of 0 indicates the phase values are distributed randomly. In experiments investigating the effects of sarcosine or nipecotic acid on step cycle phasing, no measurements were made until at least 10 min after drug application to ensure sufficient time for the drug to wash into the preparation. Addition of either drug to wild-type spinal cord preparations had no affect on left-right or extensor-flexor alternation (data not shown).

Measurements of step cycle period (defined as the interval between onset of burst n and burst n + 1) and burst duration (defined as time between onset of burst n and offset of burst n) were determined by analysis of lL2 or rL2 activity using the DATAPAC software (Run Technologies). Averages of step cycle period and burst duration were determined from all locomotor bursts that occurred once a stable pattern of locomotor-like activity had been established.

Neuronal Classification

V0 neurons are defined as the entire complement of neurons generated from the Dbx1 progenitor domain. $V0_V$ neurons derive from the ventral (Pax7$^-$) half of the Dbx1 progenitor domain and correspond to the Evx1/2$^+$ set of V0 neurons described previously in Pierani et al. (2001). $V0_D$ neurons derive from the dorsal (Pax7$^+$) half of the Dbx1 progenitor domain and correspond to D5 interneurons (Pierani et al., 2001). In the absence of additional markers for V0 interneurons, we cannot exclude that V0 neurons are divisible into further functionally distinct classes of commissural interneurons. Each of these classes is likely to be disrupted in *Dbx1* mutants.

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