LETTER

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Dual-mode operation of neuronal networks involved in left–right alternation

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All forms of locomotion are repetitive motor activities that require coordinated bilateral activation of muscles. The executive elements of locomotor control are networks of spinal neurons that determine gait pattern through the sequential activation of motor-neuron pools on either side of the body axis[1–4]. However, little is known about the constraints that link left–right coordination to locomotor speed. Recent advances have indicated that both excitatory and inhibitory commissural neurons may be involved in left–right coordination[5–7]. But the neural underpinnings of this, and a possible causal link between these different groups of commissural neurons and left–right alternation, are lacking. Here we show, using intersectional mouse genetics, that ablation of a group of transcriptionally defined commissural neurons—the V0 population—leads to a quadrupedal hopping at all frequencies of locomotion. The selective ablation of inhibitory V0 neurons leads to a lack of left–right pattern at low frequencies, mixed coordination at medium frequencies, and alternation at high locomotor frequencies. When ablation is targeted to excitatory V0 neurons, left–right alternation is present at low frequencies, and hopping is restricted to medium and high locomotor frequencies. Therefore, the intrinsic logic of the central control of locomotion incorporates a modular organization, with two subgroups of V0 neurons required for the existence of left–right alternating modes at different speeds of locomotion. The two molecularly distinct sets of commissural neurons may constrain species-related naturally occurring frequency-dependent coordination and be involved in the evolution of different gaits.

V0 neurons, characterized by the early expression of the transcription factor Dbx1 (refs 8, 9), represent a major class of commissural neurons in the ventral spinal cord, where the locomotor network is localized[2]. Previous experiments have shown that knockout of the *Dbx1* gene leads to an intermittent disruption of, but a preserved possibility for, left–right alternation during drug-induced locomotion *in vitro*[10]. These experiments also indicated an exclusive role for inhibitory commissural neurons in securing left–right alternation. Here, we tested *in vivo* and *in vitro* the possibility that transmitter-defined subsets of commissural neurons may condition left–right alternation at distinct locomotor frequencies using the $Dbx1^{loxP\text{-}stop\text{-}loxP\text{-}DTA}$ ($Dbx1^{DTA}$; DTA, diphtheria toxin A)[11] mouse strain that allows conditional ablation of V0 cells. Crossing the $Dbx1^{DTA}$ line with generic Cre mouse lines led to lethal genotypes owing to breathing impairements[12]. However, when $Dbx1^{DTA}$ mice were mated with the *E1Ngn2::Cre* line[13], where Cre is expressed in the spinal cord but has limited impact on the respiratory centres, *E1Ngn2::Cre;*$Dbx1^{DTA}$ pups survived[14]. Juvenile mutant animals (3–4 weeks) showed a pronounced quadrupedal hopping, with sequential synchronized lifting (phase values around 0 or 1) of the forelimbs followed by synchronized lifting of the hindlimbs (Fig. 1a, bottom, and Supplementary Video 1). This behaviour was never seen in age-matched wild-type mice at comparable frequencies of locomotion, in which alternating gait patterns (phase values around 0.5) dominated (Fig. 1a, top, and Supplementary Video 2).

The V0 population is composed of dorsal ($V0_D$) and ventral ($V0_V$) neuronal sub-classes that arise respectively from the dorsal ($Pax7^+$) and ventral ($Pax7^-$) subdomains of Dbx1-expressing (p0) progenitors[8,9]. We found that the E1 enhancer of Ngn2 drives Cre expression in the majority of $Dbx1^+$ progenitors of both subdomains (Fig. 1b, c). Accordingly, *E1Ngn2::Cre;*$Dbx1^{DTA}$ embryos show a marked reduction in $Dbx1^+$ progenitors and Evx1-expressing neurons, which are a known $V0_V$ contingent (Fig. 1d, e), whereas adjacent progenitors (pd6, p1 and p2a) were spared (Supplementary Fig. 1). Together, these findings indicate that the quadrupedal hopping is due to a specific elimination of both $V0_D$ and $V0_V$ neurons.

To rule out the possibility that the *E1Ngn2::Cre;*$Dbx1^{DTA}$ phenotype may result in part from the loss of V0 neurons in supraspinal structures, we used isolated spinal cords from newborn mice (Fig. 1f), in which locomotor-like activity can be induced by the exogenous application of neuro-active substances such as NMDA (*N*-methyl-D-aspartate) and serotonin, or 5-hydroxytryptamine (5-HT). In such conditions, wild-type preparations show a typical pattern of hindlimb locomotor-like activity characterized by left (L)–right (R) alternation between corresponding flexor-related lumbar (L)2 roots (LL2–RL2) and extensor-related lumbar 5 roots (LL5–RL5), and flexor–extensor alternation (LL2–LL5; RL2–RL5) (Fig. 1g, top; seen as phase values around 0.5 in circular plots in Fig. 1g, bottom). By contrast, preparations from *E1Ngn2::Cre;*$Dbx1^{DTA}$ animals (Fig. 1h, top) show rhythmic activity characterized by intrasegmental synchrony (phase values around 0 or 1) (LL2–RL2; LL5–RL5). Flexor–extensor alternation (LL2–LL5; RL2–RL5) was maintained with a larger spread of coordination around strict out-of-phase alternating activity as compared with wild-type mice ($P < 0.001$; Fig. 1h, bottom). Further confirmation of the spinal origin of the *E1Ngn2::Cre;*$Dbx1^{DTA}$ phenotype came from examining *Hoxb8::Cre;*$Dbx1^{DTA}$ animals, in which Cre recombination is restricted to spinal segments caudal to cervical segment 4 (Supplementary Fig. 2a–c)[15]. These conditional mutants showed, as did the *E1Ngn2::Cre;*$Dbx1^{DTA}$ mice, a clear hopping phenotype both *in vivo* and *in vitro* (Supplementary Fig. 2d, e and Supplementary Video 3). The hopping phenotype was complete at the hindlimb level whereas episodes of alternation were still present in the forelimbs, reflecting the rostrocaudal gradient of Hoxb8 expression. Together, these data show that major (*E1Ngn2::Cre;*$Dbx1^{DTA}$) or complete (*Hoxb8::Cre;*$Dbx1^{DTA}$) depletion of V0 neurons leads to a hopping locomotor phenotype that is due to changes in left–right alternating circuitries contained within the spinal cord. Although V0 neurons are not necessary for flexor–extensor alternation, their elimination may affect coordination because of long-range commissural projections in the lumbar spinal cord[7].

When spontaneously moving with locomotor frequencies ranging from 2–10 Hz, juvenile wild-type mice either walk (lower end of frequencies) or trot (higher end of frequencies)[16] (Fig. 2a). At higher locomotor frequencies (>10 Hz) wild-type mice might gallop[16]. In the same range of locomotor frequencies at which wild-type mice show alternating gaits (walking/trotting), V0-ablated mice consistently

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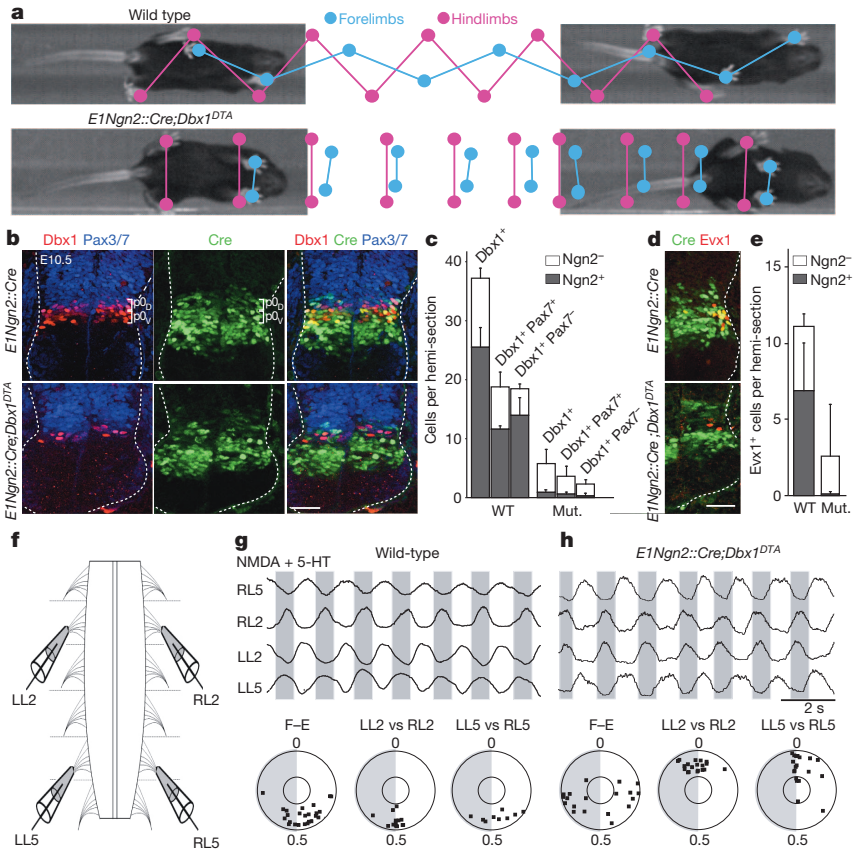

Figure 1 | Ablation of V0 neurons leads to a hopping gait. **a**, Locomotor pattern in freely moving wild-type and *E1Ngn2::Cre;Dbx1*^{DTA} mutant mice. **b**, Partitioning of Dbx1^{+} progenitors at E10.5 into dorsal (p0$_D$: Pax7^{+}) and ventral (p0$_V$: Pax7^{-}) subdomains, and co-expression of E1Ngn2::Cre, in both genotypes. Scale bar: 50 μm. **c**, Corresponding quantifications (*E1Ngn2::Cre* (wild type; WT), $N = 3$; *E1Ngn2::Cre;Dbx1*^{DTA} (mutant; Mut.) $N = 3$, mean ± standard deviation (s.d.)). **d**, Evx1^{+} V0$_V$ interneurons and E1Ngn2::Cre expression in both genotypes at E10.5. Scale bar: 50 μm. **e**, Corresponding quantifications (*E1Ngn2::Cre* (WT) $N = 3$; *E1Ngn2::Cre; Dbx1*^{DTA} (Mut.) $N = 4$, mean ± s.d.). **f**, Ventral root recording configuration for *in vitro* experiments. **g**, **h**, Top, rectified recordings of locomotor-like pattern in left (L) and right (R) lumbar (L)2 and 5 ventral roots of wild-type and *E1Ngn2::Cre;Dbx1*^{DTA} mice. Bottom, corresponding circular plots showing the phase relationships LL2–RL2 (wild-type, $N = 14$; *E1Ngn2::Cre;Dbx1*^{DTA}, $N = 19$), LL5–RL5 ($N = 9$; $N = 19$) and flexor (F)–extensor (E) ($N = 24$; $N = 20$).

showed a quadrupedal (*E1Ngn2::Cre;Dbx1*^{DTA}, Fig. 2b) or hindlimb (*Hoxb8::Cre;Dbx1*^{DTA}, Supplementary Fig. 2d) hopping gait. Similarly, isolated spinal cord preparations from *E1Ngn2::Cre;Dbx1*^{DTA} (Fig. 2c, d) and *Hoxb8::Cre;Dbx1*^{DTA} (Supplementary Fig. 2e) mice also produced hindlimb activity with left–right synchrony at all obtainable locomotor frequencies. These experiments show that ablation of V0 neurons leads to loss of left–right limb alternation along the entire locomotion frequency domain at which alternation is normally present.

Unlike in *Dbx1*-knockout mice[10], left–right alternation could not be restored in *E1Ngn2::Cre;Dbx1*^{DTA} mice by increasing the inhibitory network drive with uptake blockers of glycine and GABA (γ-aminobutyric acid) (Supplementary Fig. 3), showing that in the absence of V0 neurons normal alternating gait is not secured by other commissural pathways. These observations point to the differences between gene knockout strategies, in which targeted cells may acquire a different fate but may still be part of the network, and DTA-mediated ablation, which permanently removes the targeted cells. Moreover, the blockade of fast glycinergic and GABAergic inhibition in the presence of neuro-active drugs produced a left–right and flexor–extensor synchrony indistinguishable from that seen in wild-type mice (Supplementary Fig. 4), indicating that bilateral synchronization is mediated by a non-V0 commissural population.

The V0 population is composed of V0$_V$ and V0$_D$ subpopulations, and of excitatory and inhibitory contingents[10], raising the question of how these molecularly and transmitter-defined populations are linked, and what their individual contributions are to left–right alternation. In the mouse lumbar ventral spinal cord, Pax7-derived V0$_D$ neurons constitute two-thirds of the V0 lineage and are predominantly GABAergic or glycinergic inhibitory neurons (Supplementary Fig. 5a–d). Evx1^{+} V0$_V$ neurons constitute one-third of the V0 lineage (Supplementary Fig. 5b, c) and largely express Vglut2, the only vesicular glutamate transporter found in ventral spinal neurons[17], and no inhibitory transmitter markers (Supplementary Fig. 5d–f). Therefore, the genetic partitioning of p0 progenitors in mice leads to the production of excitatory (V0$_V$, one-thirds) and inhibitory (V0$_D$, two-thirds) subgroups of V0 neurons.

We first tested the role of the inhibitory, Pax7-derived, V0$_D$ neurons in bilateral coordination by partnering *Pax7::Cre*[18] with *Dbx1*^{DTA} mice. This cross efficiently ablated p0$_D$ progenitors (Fig. 3a), whereas the p0$_V$-derived Evx1^{+} neurons (Fig. 3a, c) and the pd6, p1 and p2 progenitors were normally produced (Fig. 3b). *Pax7::Cre;Dbx1*^{DTA} mutants die at birth from impaired breathing[12], precluding any *in vivo* investigations. Nevertheless, hindlimb locomotor-like activity could be induced in isolated spinal cords from embryonic day (E)18.5 embryos (Fig. 3d). Mutant preparations did not show any coordinated locomotor-like activity below 0.20 Hz (Fig. 3e). At medium frequencies (0.20–0.45 Hz), left–right alternation coexisted with synchronous activity in flexor-related L2 roots, whereas the extensor-related L5 roots showed synchronization. At the highest obtainable frequencies (>0.45 Hz), the flexor-related L2 ventral roots featured clear left–right alternation (Fig. 3e, middle). These trends in frequency–phase relationships were also seen when phase values were converted to a half-circle and weighted regression lines were fitted to the combined data sets (Supplementary Fig. 6a). The differences in L2 and L5 activity may reflect the greater robustness of left–right alternating circuits in the rostral compared with the caudal lumbar spinal cord[19]. These experiments show that the inhibitory V0$_D$ neurons are needed for left–right alternation at low frequencies but dispensable for alternation at the highest locomotor frequencies. In the absence of the V0$_D$ population, the remaining commissural neurons do not sustain persistent alternation at medium-range frequencies, leading to the presence of both intrasegmental alternation and synchrony.

We next assessed the role of the excitatory V0$_V$ commissural neurons by crossing *Dbx1*^{DTA} with *Vglut2::BAC-Cre* (*Vglut2::Cre*) mice[17]. Cre expression was found in one-third of Dbx1-derived V0 neurons, predominantly in Evx1^{+} V0$_V$ neurons (Supplementary Fig. 5c–f). Accordingly, *Vglut2::Cre;Dbx1*^{DTA} animals lacked most Evx1^{+} V0$_V$

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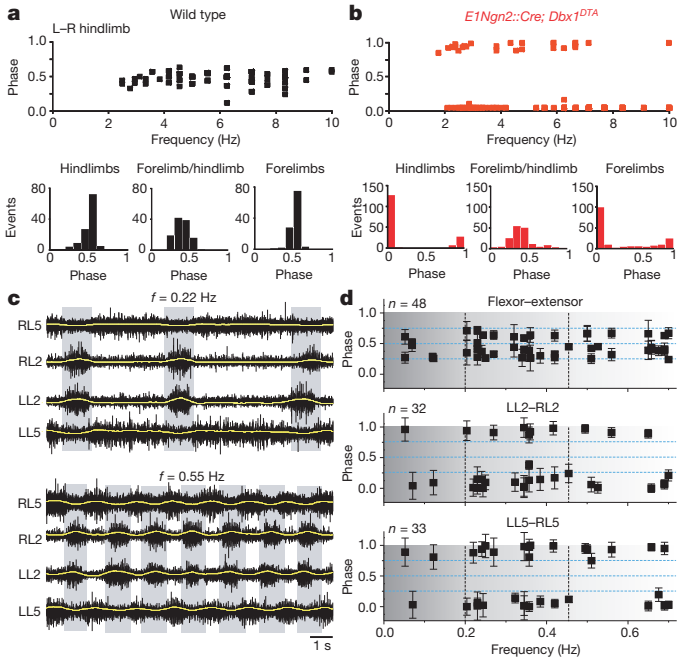

Figure 2 | V0-deleted mice hop at all locomotor frequencies. **a**, **b**, Locomotor gait in wild-type and *E1Ngn2::Cre;Dbx1*^{DTA} mice. Top, frequency–phase relationship of wild-type (**a**; $N = 3$) and *E1Ngn2::Cre;Dbx1*^{DTA} (**b**; $N = 4$) mice. Bottom, phase histograms between indicated pairs of limbs. **c**, Ventral root recordings during drug-induced locomotor-like activity in postnatal day (P)0–P1 *E1Ngn2::Cre;Dbx1*^{DTA} preparations at different frequencies (obtained by changing the concentrations of locomotor-inducing drugs). Raw ventral root recordings shown in black with superimposed rectified and integrated signals in yellow (same in Figs 3d and 4d). **d**, Corresponding frequency–phase plots where each square represents the mean phase of a significantly oriented phase vector ($N = 11$). Error bars show angular dispersion. The different scales of grey indicate low- (<0.2 Hz), intermediate- (0.20–0.45 Hz) and high-frequency (>0.45 Hz) regimes here and in Figs 3e and 4e.

neurons (Supplementary Fig. 7a and Fig. 4a) but there was no change in the number of the adjacent Di6, V2a and V1/V0$_D$ neurons (Supplementary Fig. 7b, c). We did not exclude a possible effect on the small population of Dbx1-derived Pitx2-expressing neurons[20]. Importantly, the main respiratory populations were spared, allowing *in vivo* analysis. Juvenile mutant animals showed either a left–right alternating or hopping hindlimb gait in a frequency-dependent manner: an alternating pattern was present at low locomotor frequencies (2–4 Hz) and a hopping gait at high frequencies (4–10 Hz) (Fig. 4b, c and Supplementary Video 4). The forelimbs maintained left–right alternation at all speeds. This frequency-dependent locomotor phenotype was recapitulated *in vitro*, with locomotor-like activity showing a hindlimb 'hopping' pattern at the higher and alternation at the lower frequencies (Fig. 4d, e and Supplementary Fig. 6b). At intermediate frequencies, flexor-related L2 and extensor-related L5 ventral roots showed either alternation or synchrony (Fig. 4d, e). Flexor–extensor alternation was close to normal at all frequencies. Blockade of fast inhibition in these animals resulted in bilateral synchronization (Supplementary Fig. 4c). Thus, glutamatergic V0 neurons are necessary for hindlimb alternation at high locomotor frequencies but dispensable at low locomotor frequencies. By contrast, at the forelimb level, V0$_V$ neurons are dispensable for alternation at all frequencies, suggesting that V0$_D$ neurons may be the dominant alternating system in the forelimb locomotor network.

Using both *in vivo* and *in vitro* experiments, we have identified V0 commissural neurons as necessary for alternating gaits in mice. Together with previous studies on Dbx1-independent commissural neurons[21,22], our study points to a role for distinct commissural pathways in ensuring different modes of left–right locomotor coordination. V0 neurons seem to be the major executors necessary for left–right alternating gaits, whereas other non-V0 commissural neurons promote synchrony and presumably secure galloping at high speeds of locomotion in intact mice.

The V0 alternating network relies on distinct molecularly defined populations that enforce alternation at the low and high ends of the alternating locomotor frequency domain. Studies in newborn rodents[5,7] have revealed that contralateral inhibition of motor neurons can be mediated both directly, by inhibitory commissural neurons, and indirectly, via excitatory commissural neurons acting on local inhibitory interneurons. The present study elucidates the genetic underpinning and shows the functional expression of this dual organization of the alternating locomotor network, and opposes the generally held notion that left–right alternation is mediated by inhibitory commissural neurons alone[1,10,23]. The implication is that the configuration of the left–right alternating locomotor network changes with speed. The inhibitory V0$_D$ neurons are recruited first, during slow locomotion, followed by excitatory V0$_V$ neurons recruited at higher frequencies. It is conceivable that at intermediate frequencies of locomotion the two systems are active simultaneously. Such speed-dependent changes in recruitment order have been described in zebrafish for classes of excitatory interneurons driving motor neurons during swimming movements[24,25]. The network switch described here is in accord with findings in mice

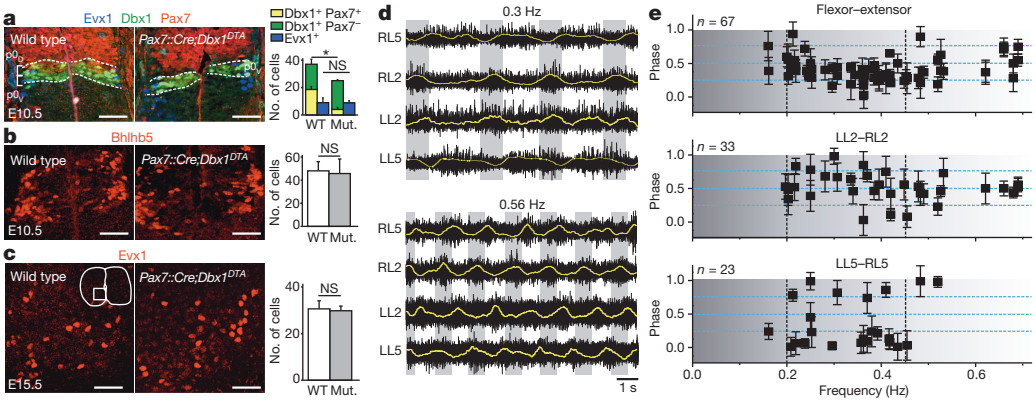

Figure 3 | V0$_D$-deleted mice show a lack of alternation at low frequencies and alternation at high frequencies. **a**, Pax7-expressing dorsal Dbx1$^+$ progenitors (p0$_D$) are ablated in *Pax7::Cre;Dbx1*^{DTA} mutants (Mut.) as compared with wild type (WT), whereas the ventral p0 (p0$_V$, Pax7$^-$) neural progenitors and the V0$_V$ neurons expressing Evx1 are spared ($N = 3$ in each group, mean ± s.d.). NS, not significant. **b**, The expression of Bhlhb5, a marker for pd6, p1 and p2a progenitors at E10.5 (ref. 30), is preserved in *Pax7::Cre;Dbx1*^{DTA} mutant mice (wild type, $N = 4$; *Pax7::Cre;Dbx1*^{DTA}, $N = 4$; mean ± s.d.) **c**, V0$_V$ Evx1$^+$ neurons are spared in *Pax7::Cre;Dbx1*^{DTA} mutant mice at E15.5 (wild type, $N = 3$; *Pax7::Cre;Dbx1*^{DTA}, $N = 3$; mean ± s.d. **a**–**c**, Scale bars: 50 μm. **d**, Ventral root recordings during drug-induced locomotor-like activity in E18.5 *Pax7::Cre;Dbx1*^{DTA} preparations at different frequencies. **e**, Corresponding frequency–phase plots ($N = 21$). Error bars show angular dispersion.

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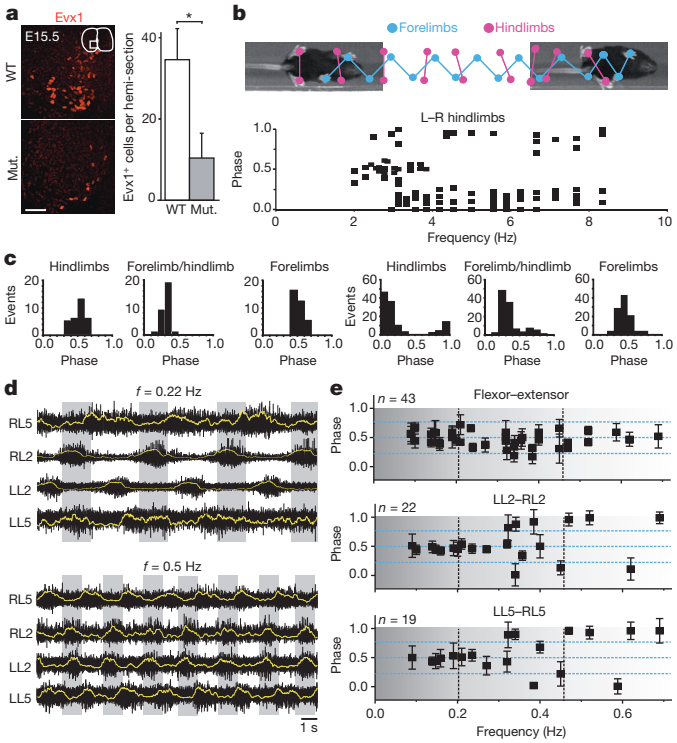

Figure 4 | Deletion of excitatory $V0_V$ neurons causes hopping at high locomotor frequencies but preserves alternation at lower frequencies. **a**, $V0_V$ ($Evx1^+$) neurons in E15.5 wild-type (WT; $N = 3$) and *Vglut2::Cre; Dbx1*^{DTA} mutant (Mut.; $N = 3$) lumbar spinal cord (scale bar: 50 μm; mean ± s.d.). **b**, Locomotor pattern at medium to high frequencies and frequency–phase relationship of hindlimbs (bottom) in *Vglut2::Cre;Dbx1*^{DTA} mice. **c**, Phase histograms between the indicated pairs of limbs at low (2–4 Hz; three panels to the left) and high (4–10 Hz; three panels to the right) frequencies. **d**, Ventral root recordings during drug-induced locomotor-like activity in P0–P1 *Vglut2::Cre;Dbx1*^{DTA} preparations at different frequencies. **e**, Corresponding frequency–phase plots ($N = 12$). Error bars show angular dispersion.

that ipsilaterally projecting V2a excitatory neurons project to $V0_V$ neurons[26] and that V2a-ablated mice hop[27] at higher frequencies. These findings suggest that ensembles of neurons are selected as modules[28] at different speeds of locomotion.

The Dbx1/Evx1 transcription factors seem to be phylogenetically conserved[29], and a modular system similar to the one described here may be found in other vertebrates with alternating gait. Moreover, during evolution bipedal and quadrupedal hopping have developed in a number of species and some strains of rodents, suggesting that this change in locomotor behaviour may be carried by a loss, partial or complete, of the V0-determined alternating system.

METHODS SUMMARY

All experiments were approved by the local ethical committee. For full details of methods used, including detailed information about mice lines, the *in vivo* and *in vitro* locomotor recordings and analysis, statistical analysis and protocols for immunohistochemical analysis, see Methods.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions O.K., A.E.T., L.B. and J.B. contributed to the conception and design of the study. A.E.T. performed electrophysiological experiments, J.B. performed anatomical experiments and both analysed the data. L.B. carried out and analysed the *in vivo* experiments. A.P. engineered the *Dbx1*^{DTA} mice and detected the hopping phenotype in the *E1Ngn2::Cre;Dbx1*^{DTA} mice. G.F. provided mice and fixed tissue. O.K. supervised all aspects of the work. All authors discussed the results and participated in writing the manuscript.

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METHODS

All experiments were approved by the local ethical committee and performed in accordance with European guidelines for the care and use of laboratory animals. The following transgenic lines have been described previously: *Pax7::Cre*[18], *E1Ngn2::Cre*[13], *Vglut2::Cre*[17], *Dbx1*$^{lox\text{-}STOP\text{-}lox\text{-}DTA}$ (*Dbx1*^{DTA})[11], *Dbx1*^{nlsLacZ} (ref. 9); *Glyt2-GFP*[31,32]; *GAD67-GFP*[32,33], *Hoxb8::Cre*[15]. All animals were genotyped after the experiments with gene-specific primers.

***In vivo* locomotor analysis.** All *in vivo* data were recorded using a TSE MotoRater system that captures the natural movement of animals on a runway. The animals were allowed to walk or run freely in a glass-walled corridor and recorded by high-speed camera at 100–200 frames per second. For each mouse, runs from at least three independent recordings were analysed using the Clickjoint software. The first and the last step in a run and episodes with frequencies lower than 2 strides per second were not included in the analysis. Phase values were calculated as the time of the swing onset of a limb related to the step cycle length of the reference limb in that particular step cycle. One step cycle corresponds to the time from swing onset until the next swing onset.

***In vitro* experiments.** Mice aged E18.5 (*Pax7::Cre; Dbx1*^{DTA}) or newborn mice aged 1–2 days were used in all experiments. The embryonic state was called E0.5 on the morning of plug detection. For analyses at E18.5, fetuses were surgically removed from pregnant mice, cooled, decapitated and eviscerated before the brainstem and spinal cord were isolated in ice-cold low calcium Ringer's solution (oxygenated 95% O_2, 5% $C0_2$) that contained (in mM): 111 NaCl, 3 KCl, 11 glucose, 25 $NaHCO_3$, 3.7 $MgSO_4$, 1.1 KH_2PO_4, 0.25 $CaCl_2$ (pH 7.4). Newborn animals were anaesthetized with isoflurane and dissected as described previously[34]. The isolated spinal cord was transferred to a recording chamber that was continuously perfused with normal Ringer's solution and contained (in mM): 111 NaCl, 3 KCl, 11 glucose, 25 $NaHCO_3$, 1.25 $MgSO_4$, 1.1 KH_2PO_4, 2.5 $CaCl_2$ oxygenated 95% O_2, 5% CO_2 to obtain a pH of 7.4. All recordings were done at room temperature (22–24 °C).

***In vitro* recording of locomotor activity and analysis.** Locomotor activity was recorded with suction electrodes attached to the L2 and L5 lumbar roots on either side of the cord. The ventral root activity was band-pass filtered at 100 Hz to 1 kHz. The signal was sampled using Axoscope 10 (Molecular Devices) at 1–2 kHz. Data points for analysing cycle periods and phases were taken after the locomotor activity had stabilized 10–15 min after the initial burst of activity. Locomotor wave onsets were automatically detected and calculated based on the intersection between the smoothed traces with a horizontal line running through the centre of the trace. The cycle periods of the reference trace were normalized to a scale of 360 degrees, with the onset of a locomotor burst in the reference ventral root corresponding to a phase value of 0° and the onset of the next burst corresponding to a phase value of 360°. The phase delay of the period cycle onset with respect to the reference was calculated for test traces. Epochs of at least 20 consecutive phase values were taken from a sample of at least 70 consecutive phase values. Flexor–extensor and left–right coordination were evaluated with circular statistics (see ref. 34), in which the vector direction gives the preferred mean phase of the activity, and the length of the vector (**r**) the precision of the phase. *P* values greater than 0.05 as determined by Rayleigh's test were considered non-significant. The angular dispersions (a variance measure) were calculated from **r** values and plotted as error bars[35]. Values for frequency-phase plots in the *Vglut2::Cre;Dbx1*^{DTA} and *Pax7::Cre;Dbx1*^{DTA} mice were also generated using the Spinalcore software[36] (using Morlet wavelet algorithms) to obtain frequencies and phases. The condition for inclusion in these frequency-phase plots was that each of the samples (n) involves at least two significantly oriented vector relationships between activities in the ventral roots (LL2–RL2 and/or LL5–RL5 (left–right), and LL2–LL5 and/or RL2–RL5 (flexor–extensor)). The Watson–Williams test for circular data was used to compare conditions[35].

The different locomotor frequencies for the *in vitro* experiments were obtained by varying the concentrations of locomotor-inducing drugs (5-HT in combination with NMDA)[34]. We classified frequency-phase data in groups according to their locomotor frequency: high frequency, $\langle f \rangle > 0.45$ Hz; medium frequency, $0.45 \geq f \geq 0.2$ Hz; and low frequency, $f < 0.2$ Hz. These arbitrary limits were set to include half and double the average locomotor frequencies obtained during drug-induced locomotor-like activity wild-type mice.

Linear fits were produced to correlate changes in the phase to changes in the locomotor frequency (Supplementary Fig. 6). To make these calculations possible, the circular data in Fig. 3e and Fig. 4e were converted to semi-circular coordinates assuming a central symmetry of data and bimodality for the two opposite states: alternation (0.5 turn) and synchrony (0.0 turn). Thus, concentration of the angle distribution within the 0.0–0.5 range forces the direction of angular variance to contribute to the linear fit[37]. Having done this conversion, the weighted linear fits using means and semi-circular dispersion data were calculated by the least mean squares method on circular data (Supplementary Fig. 6). Significance of the correlation was additionally assessed with the Spearman's rank correlation, r_s, a test not requiring normal distribution of the two variables.

Drugs. NMDA was used in combination with 5-HT to induce locomotor-like activity *in vitro*. $GABA_A$ and glycine receptors were blocked with picrotoxin (10 μM) and strychnine (0.5 μM), respectively. Sarcosine (100 μM) and nipecotic acid (120 μM) were used to block glycine and GABA uptakes, respectively. All drugs were purchased from Sigma.

Immunohistochemistry. Whole embryos (E10.5) or isolated lumbar spinal cords (E15.5 to newborn) were fixed for 2 h in 4% (wg/vol) paraformaldehyde in phosphate-buffered saline (PBS), rinsed in PBS, cryoprotected in 30% (wg/vol) sucrose in PBS overnight, and embedded in OCT mounting medium. Transverse sections (16-μm thick for E10.5/E12.5, or 20 μm for E15 to postnatal day 1) were obtained on a cryostat. Sections were incubated overnight at 4 °C with one or several of the following primary antibodies diluted in PBS supplemented with 5% (vol/vol) fetal bovine serum and 0.5% (wg/vol) Triton X-100: rabbit anti-Dbx1 (1:10,000), chicken anti-GFP (Aves Labs 1020; 1:2,000), chicken anti-β-Gal (abcam 9361; 1:1,000), guinea pig anti-Evx1 (1:4,000), goat anti-Pax3/7 (Santa Cruz 7748, 1:200), rabbit anti-Cre (1:8,000, gift from G. Shutz[17]), guinea pig anti-Bhlhb5 (1:20,000, gift from B. Novitch[38]), guinea pig or rabbit anti-Lbx1 (1:10,000, gift from C. Birchmeier[39], rabbit anti-GABA (1:1,000, Sigma), sheep anti-Chx10 (1:500, Chemicon), and rabbit anti-Pax2 (1:300, Covance). Secondary antibodies were obtained from Jackson Immunoresearch or Invitrogen and incubated for 1 h at room temperature. Slides were rinsed, mounted in Vectashield medium and scanned on a LSM5 confocal microscope (Zeiss Microsystems) using a ×20 or a ×40 objective. Multiple channels were scanned sequentially to prevent fluorescence bleedthrough and false-positive signals. A contrast enhancement and a noise reduction filter were applied in Adobe Photoshop for publication images.

Cellular counts. For experiments at E10.5, five to ten non-adjacent cryo-sections, encompassing 300 to 600 μm of the hindlimb bud area, were counted. For experiments at E15.5–E16.5, five to ten non-adjacent sections spanning the rostral lumbar spinal cord (T13 to L3) were counted. Neurons expressing the makers of interest were counted on the same side, using raw z-stack confocal images obtained with the ×40 objective and spanning the entire thickness of the sections. Counts were done manually with the help of the cell-counter plug-in in ImageJ. Cellular counts per section were averaged (or expressed as percentages) per individual animal, and the grand mean (or grand mean percentage) ± standard deviation was calculated across animals to produce cells per hemi-section (or percentage) column plots summarizing anatomical staining in the figures.

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