In Mice Lacking V2a Interneurons, Gait Depends on Speed of Locomotion

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Many animals are capable of changing gait with speed of locomotion. The neural basis of gait control and its dependence on speed are not fully understood. Mice normally use a single “trotting” gait while running at all speeds, either over ground or on a treadmill. Transgenic mouse mutants in which the trotting is replaced by hopping also lack a speed-dependent change in gait. Here we describe a transgenic mouse model in which the V2a interneurons have been ablated by targeted expression of diphtheria toxin A chain (DTA) under the control of the *Chx10* gene promoter (Chx10::DTA mice). Chx10::DTA mice show normal trotting gait at slow speeds but transition to a galloping gait as speed increases. Although left–right limb coordination is altered in Chx10::DTA mice at fast speed, alternation of forelegs and hindlegs and the relative duration of swing and stance phases for individual limbs is unchanged compared with wild-type mice. The speed-dependent loss of left–right alternation is recapitulated during drug-induced fictive locomotion in spinal cords isolated from neonatal Chx10::DTA mice, and high-speed fictive locomotion evoked by caudal spinal cord stimulation also shows synchronous left–right bursting. These results show that spinal V2a interneurons are required for maintaining left–right alternation at high speeds. Whether animals that generate galloping or hopping gaits, characterized by synchronous movement of left and right forelegs and hindlegs, have lost or modified the function of V2a interneurons is an intriguing question.

Introduction

Many mammalian species can generate a set of distinct gaits for moving over diverse terrain and at various speeds (Hildebrand, 1989). Both spinal and intact cats shift from trotting to galloping with increased treadmill speed (Forssberg et al., 1980), showing that gait changes can be performed by isolated spinal locomotor networks and that locomotor speed is an important component in determining gait. Mice use a trotting gait, with regular alternation of opposing left and right limbs, during both over-ground and treadmill locomotion, over their entire range of speeds (Herbin et al., 2007). Mice with mutations in *ephrinB3/EphA4* hop at all speeds (Kullander et al., 2003), suggesting that the inability of mice to switch gait at various locomotor speeds is limited by neural rather than biomechanical constraints.

The spinal circuits that govern gait changes at different speeds have not been characterized in mammals. Larval zebrafish, juvenile reef fishes, frogs, and salamanders alternate fins or limbs during slow speeds but use synchronous limb movements or axial

body undulations to propel themselves at high speeds (Nauwelaerts, 2002; Thorsen et al., 2004; Hale et al., 2006; Chevallier et al., 2008). In zebrafish, different sets of spinal neurons become active during slow and fast swimming (Kimura et al., 2006; McLean et al., 2007, 2008; Liao and Fetcho, 2008). The speed-dependent recruitment of neurons has been confirmed for two excitatory interneuron types (McLean et al., 2008). One of these interneuron subtypes, called CiD, is marked by expression of the *alx* gene (Kimura et al., 2006). However, the precise function of CiD interneurons in the zebrafish spinal cord is not known.

In the mouse spinal cord, a class of interneurons called V2a neurons expresses *Chx10*, a homolog of the zebrafish *alx* gene (Al-Mosawie et al., 2007; Lundfald et al., 2007; Peng et al., 2007). The genetic cascade for the development of *alx*-expressing interneurons in the zebrafish and V2a interneurons in the mouse spinal cord has been conserved during evolution (Peng et al., 2007). In the mouse, genetic ablation of V2a neurons [Chx10::DTA mice (Crone et al., 2008)] results in deficits in consistent left–right alternation during fictive locomotion (Crone et al., 2008). How the function of V2a interneurons might change with locomotor speed in adult mice was not examined, because the BL6 strain of mice, expressing diphtheria toxin A chain (DTA) in V2a neurons, invariably died shortly after birth as a result of respiratory distress (Crone et al., 2008).

Here we make use of genetic variation in different mouse strains to avert the neonatal lethality after targeted ablation of V2a neurons. In the mixed ICR/BL6/129 genetic background in which wild-type mice maintain left–right alternation through all speeds of locomotion, we find that left–right alternation is pro-

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gressively lost and replaced by synchronous galloping as Chx10::DTA mice run faster. We show that the switch in gait at faster speeds is a function of the loss of spinal V2a interneurons because, in isolated spinal cords from neonatal Chx10::DTA mice, normal left–right alternation is also weakened and replaced by left–right synchrony at higher frequencies of motor activity compared with lower frequencies. These data show that V2a interneurons in the lower spinal cord are responsible for maintaining left–right alternation but only at faster speeds of locomotion.

Materials and Methods

Ethics statement. All animal care was performed in accordance to the University of Chicago Animal Care and Use Committee and the Cornell Institutional Animal Care and Use Committee, in accordance with National Institutes of Health guidelines.

Transgenic animals. Generation of Chx10::DTA mice and genotyping protocols have been described previously (Crone et al., 2008). Breeding was performed as follows: pr^m-Cre; chx10::LNL::DTA male mice, on a mixed C57BL/6 and 129/SvEv (embryonic stem cell component) genetic background, were bred to ICR females to produce heterozygous offspring with a single copy of the recombined *chx10::DTA* allele (Chx10::DTA mice) or homozygous littermates with two wild-type *chx10* alleles (wild-type littermates). Less than 2% of offspring from this breeding contain a nonrecombined *chx10::LNL::DTA* allele, and these mice were not included in this study.

Immunohistochemistry. Neonatal [postnatal day 0 (P0)] pups were anesthetized and perfused with 4% paraformaldehyde, and brains and spinal cords were dissected out, postfixed for 2 h in 4% paraformaldehyde, and washed overnight in PBS. Tissue was cryoprotected in 30% sucrose for 2–4 h and mounted in OCT medium (Tissue Tek) for cryosectioning. Transverse sections (14 μ m) of lumbar spinal cord or medulla were stained with antibodies to Chx10 (guinea pig, 1:10,000) (Thaler et al., 1999) or GATA-2/3 (guinea pig, 1:8000) according to the methods described by Peng et al. (2007). Biotinylated secondary antibodies (donkey, 1:500) followed by ABC amplification (Vector Laboratories) and DAB color reaction (Sigma) were used to visualize staining. Slides were dehydrated in graded concentrations of ethanol and then xylenes and were mounted in Permount media (Thermo Fisher Scientific). Images were obtained with a Zeiss Axioplan II microscope and a Zeiss Axiocam digital camera and were analyzed with OpenLab 3.1 software (Improvision).

AChE histochemistry. Histochemical staining for AChE was performed on hindbrain sections to identify those containing the nucleus ambiguus. Slides were incubated at 37°C for 7 h in a solution containing 0.2 mM ethopropazine-HCl, 10 mM glycine, 2 mM cupric sulfate, 65 mM sodium acetate, pH 5.5, and 4 mM acetylthiocholine iodide. The color reaction was then performed in 1.25% sodium sulfide, pH 6.0, for 8 min. Slides were rinsed eight times in PBS, dehydrated in graded concentrations of ethanol and then xylenes, and mounted in Permount media (Thermo Fisher Scientific).

Neuron counts. The number of neurons positive for Chx10 or GATA-3 nuclear staining was counted on the left and right sides of 10–12 lumbar sections (14 μ m) for four wild-type and four Chx10::DTA animals, and the average number of neurons per hemisection was calculated for each animal. The number of neurons positive for Chx10 or GATA-3 nuclear staining was counted on the left and right sides of five to six medulla sections (14 μ m) adjacent to sections containing the nucleus ambiguus (as determined by AChE histochemistry) for three wild-type and three Chx10::DTA animals, and the average number of neurons per hemisection was calculated for each animal.

Gait analysis. The DigiGait instrument (Mouse Specifics) was used to analyze gait in 11 Chx10::DTA and 11 wild-type littermates aged P24–P25. The hindpaws and forepaws of mice were painted with red food coloring (McCormick) to enhance visual contrast. Each animal was allowed to run on a clear treadmill belt (with a digital camera underneath to record paw placement) at speeds of 15, 25, 35, 45, 55, 65, 75, 85, and 95 cm/s. Mice were placed on a stationary treadmill, which was then accelerated to the test speed. Ten trials were attempted at each speed, and

successful trials (in which the animal was able to maintain treadmill speed for >3 s) were counted and recorded with digital video (135 frames/s). Mice were rested at least 10 s between trials (of ~5 s each) and at least 60 min after 30 trials to prevent tiring. For each animal, one trial (500 frames of video) at each speed was selected for analysis in which the animal maintained constant position on the treadmill belt. DigiGait software was used to identify stance and swing phases for all four limbs in every video frame and to measure average values for stride length and stride frequency, as well as duration of stride, stance, and swing for each limb. Gait diagrams were produced using Excel (Microsoft) based on frame-by-frame stance and swing data provided by DigiGait software. The hindlimb phase was calculated for each step by dividing the time between the start of the right and left hindlimb stance divided by the duration of the right hindlimb step cycle (see Fig. 4A). Circular analysis of hindlimb phase was performed by representing each step as a unit vector with radius (R) = 1 and direction determined by the phase value (radians = $2\pi \times$ phase value). Each trial is represented by a vector (whose endpoint is represented as a dot in circular plots) that is the average vector for the steps in that trial. Trial vectors with R values near 1 (outer circle) represent trials in which all the steps have a consistent phase value, whereas trials with R values near 0 (center of circle) show no preferred phase value. Group vectors were averages of the vectors for all mice that attained a given speed. Statistical comparisons of R values were made using the Mann–Whitney U test as described by Zar (2000).

Whole-cord preparations. Experiments were performed with neonatal (P0–P2) Chx10::DTA mice or wild-type littermates. The dissection was performed similar to that described previously (Zhong et al., 2007). Briefly, neonatal mice were decapitated and eviscerated in cold oxygenated (95% O₂ and 5% CO₂) low-calcium Ringer's solution composed of the following (in mM): 111 NaCl, 3.08 KCl, 25 NaHCO₃, 1.18 KH₂PO₄, 3.5 MgSO₄, 0.25 CaCl₂, and 11 glucose. The spinal cord, extending from T8/T9 to S3/S4, was then dissected out and pinned ventral side up in a recording chamber superfused with oxygenated normal Ringer's solution composed of the following (in mM): 111 NaCl, 3.08 KCl, 25 NaHCO₃, 1.18 KH₂PO₄, 1.25 MgSO₄, 2.52 CaCl₂, and 11 glucose. In some experiments, the caudal end of the spinal cord was dissected, and locomotor-like activity was reliably induced by stimulating the entire caudal end of the spinal cord (Whelan et al., 2000; Gordon and Whelan, 2006; Zhong et al., 2007). Constant-current stimulus trains (0.1–1 mA, 2–2.5 Hz for 15–30 s) were delivered through a suction electrode. Ventral root recordings were bandpass filtered (100 Hz to 1 kHz) and recorded using an alternating current amplifier (model 1600; A-M Systems). All experiments were performed at room temperature.

Ventral root recordings. Suction electrodes were placed on the left (lL2) and right (rL2) L2 and left (lL5) and right (rL5) L5 ventral roots. Locomotor activity was evoked by perfusing Ringer's solution containing a combination of NMDA (4–10 μ M; Sigma) and serotonin creatine sulfate (5-HT) (4–10 μ M). No pronounced differences in locomotor-like activity were observed over the postnatal ages used (P0–P2). Rhythmic burst activity from ventral roots was bandpass filtered (100 Hz to 1 kHz). Locomotor-like activity was analyzed using two different methods: (1) statistical measure of the lL2/rL2 and lL2/lL5 coordination by using circular statistics and (2) autocorrelation analysis from L2 ventral roots and cross-correlation analysis from lL2/rL2 and lL2/lL5 ventral roots.

Circular analysis. Circular statistics were used to determine the significance of the phasing relationship between left–right and flexor–extensor motor bursts (Kjaerulff and Kiehn, 1996; Zar, 2000). Clampfit 9.0, Excel, and Spike 2 (Cambridge Electronic Design) were used for data analysis. The direction of a vector represents the average preferred phase of the ventral root motoneuron burst, whereas its length, R , is a measure of statistical significance of the preferred phase. Vectors with a phase of 0.5 or 0 corresponded to alternation or synchronization between the two ventral roots. p values for the significance of R were calculated as described by Kjaerulff and Kiehn (1996) and Zar (2000).

Correlation analysis. The ventral root extracellular data were integrated and smoothed with a time constant of 0.2 s and analyzed using custom-written programs in Spike 2 (Cambridge Electronic Design). The phase relationship between the flexor and extensor activity was quantified by cross-correlation analysis, whereas the rhythmicity of the

flexor bursts was tested by autocorrelation analysis. At least 10 min of data were analyzed for each measurement. These were subdivided into time segments of 20 s each for the correlation analyses. Autocorrelations or cross-correlations were computed for at least 20 time segments. The mean and SE values were obtained from the entire set. Ventral root activity was considered significantly rhythmic if the peak next to the center of the average autocorrelation exceeded four times the SE of the entire set (Kremer and Lev-Tov, 1997). Ventral root activity from one root was considered significantly correlated to the opposite ventral root if the central peak of the average cross-correlation exceeded four times the SE (Kremer and Lev-Tov, 1997). A positive value for the peak near 0 in the cross-correlation indicates that the two signals are relatively in-phase, whereas a negative value indicates that they are relatively out-of-phase. The exact phase relationship was measured by the circular analysis.

Results

Postnatal survival of *Chx10::DTA* mice is strain dependent

The phenotypic variability of transgenic mice is often affected by genetic variability among different mouse strains. In our previous study (Crone et al., 2008), all the progeny of wild-type inbred BL6 dams that receive a copy of the recombinant *Chx10::DTA* allele from male BL6/129 mice died before 3 d of postnatal life, likely attributable to respiratory difficulty (for details of breeding strategies and various mouse strains, see Materials and Methods). We asked whether the genetic background of the dam could improve the survival of progeny that carry a copy of the recombinant *Chx10::DTA* allele. We tested the survival of progeny from dams of the ICR strain mated to BL6/129 males. In survival studies, 40% (31 of 78) of the progeny of ICR dams that carried one copy of the *Chx10::DTA* allele survived past 3 weeks of age. The other 60% progeny of the ICR dams died within 3 d of birth. These data suggest that genetic variability between the BL6 and ICR dams is sufficient to overcome neonatal lethality in a significant proportion of mice that express DTA in V2a neurons.

We asked whether the survival of the progeny of ICR dams resulted from variability in the expression of DTA and incomplete ablation of V2a neurons. We counted the number of surviving V2a neurons in the lumbar spinal cord, as well as the medulla of newborn progeny of ICR and BL6 dams mated to *Chx10::DTA* BL6/129 male mice. The lumbar cord and medulla are the sites of locomotor and respiratory central pattern generators (CPGs), respectively. Progeny of ICR dams show an almost complete ablation of Chx10 neurons in the lumbar spinal cord (Fig. 1) (40.3 ± 2.1 cells per hemisection in wild type, 0.88 ± 0.07 cells per hemisection in *Chx10::DTA*; $p = 0.0003$) and in the medulla at the level of preBötzinger complex, in which respiratory rhythm is generated (Fig. 1) (111.3 ± 6.9 cells per hemisection in wild type, 2.2 ± 0.3 cells per hemisection in *Chx10::DTA*; $p = 0.004$). Next we asked whether the loss of V2a neurons resulted in an unexpected, compensatory change in other cell types.

Figure 1. *Chx10::DTA* pups from ICR dams lack Chx10 neurons in the spinal cord and medulla but have a normal complement of GATA-3 neurons. **A–D**, Transverse lumbar spinal cord sections from P0 wild-type (**A, B**) and *Chx10::DTA* (**C, D**) pups stained with antibodies to Chx10 (**A, C**) or GATA-3 (**B, D**). **E–H**, Transverse sections of the medulla at the level of the nucleus ambiguus from P0 wild-type (**E, F**) and *Chx10::DTA* (**G, H**) pups stained with antibodies to Chx10 (**E, G**) or GATA-3 (**F, H**). **I**, The number of Chx10 and GATA-3 cells were counted in lumbar spinal cord sections in *Chx10::DTA* mice ($n = 4$) and wild-type littermates ($n = 4$) (mean ± SEM; $p < 0.001$ for Chx10, $p > 0.10$ for GATA-3). **J**, The number of Chx10 and GATA-3 cells were counted in the medulla (at the level of the nucleus ambiguus) in *Chx10::DTA* mice ($n = 3$) and wild-type littermates ($n = 3$) (mean ± SEM; $p < 0.005$ for Chx10, $p > 0.10$ for GATA-3).

We examined expression of the transcription factor GATA-3 in *Chx10::DTA* and wild-type animals at P0. GATA-3 marks the V2b interneurons in the ventral spinal cord and medulla that are derived from the P2 progenitor domain, like the V2a Chx10 interneurons (Peng et al., 2007). We did not detect any change in the number or location of GATA-3 interneurons in the lumbar spinal cord or medulla of *Chx10::DTA* mice compared with wild-type littermates (Fig. 1) (spinal cord: 20.5 ± 0.6 cells per hemisection in wild type, 21.2 ± 0.8 cells per hemisection in *Chx10::DTA*, $p = 0.50$; medulla: 99.9 ± 7.7 cells per hemisection in wild type, 93.8 ± 7.2 cells per hemisection in *Chx10::DTA*, $p = 0.60$). These data demonstrate that efficiency and selectivity of ablation of Chx10 neurons in ICR dams is comparable in the progeny of BL6 dams that we reported previously, in which ablation of the Chx10 neurons did not change the number of *Evx1*⁺ (V0), *En1*⁺ (V1), *GATA-3*⁺ (V2b), and *Sim1*⁺ (V3) interneuron subtypes or *HB9*⁺ motor neurons (Crone et al., 2008). Thus, those *Chx10::DTA* mice that are derived from ICR dams and survive to adulthood are a suitable model to test the functions of V2a neurons in adult locomotion.

Locomotor activity in the absence of V2a interneurons

Chx10::DTA mice that died within the first 3 d of birth were indistinguishable during that period from those that ultimately survived. Within the first week, the surviving *Chx10::DTA* mice

Figure 2. Chx10::DTA mice are smaller than littermates but adjust stride parameters normally in response to changes in treadmill speed. **A**, P24–P25 wild-type ($n = 11$) and Chx10::DTA ($n = 11$) mice were tested for their ability to run at treadmill speeds from 15 to 95 cm/s, at 10 cm/s intervals. The number of mice able to maintain constant speed for at least one trial of 10 is shown. **B**, **C**, The DigiGait system was used to detect paw placement on a clear

were significantly smaller than their littermates that did not inherit a recombinant *Chx10::DTA* allele. By P24–P25, Chx10::DTA mice weigh 40% less than their wild-type littermates (wild type, 17.67 ± 0.46 g, $n = 11$; Chx10::DTA, 10.72 ± 0.64 g, $n = 11$). No obvious differences in the home-cage locomotor activity were noticeable between Chx10::DTA mice and wild-type controls. Chx10::DTA animals appeared to walk normally and were able to engage in all normal activities.

Because V2a-like interneurons have been implicated in slow as well as fast locomotor activity in the zebrafish (McLean et al., 2008), we tested the ability of Chx10::DTA mice to run on a treadmill at various speeds. Age P24–P25, Chx10::DTA and wild-type littermates were allowed to run at constant speed on a clear treadmill belt while digital video was recorded from underneath the treadmill to mark when each of the four limbs was in contact with the treadmill belt. DigiGait software was used to measure the average stride length and frequency for each limb, as well as the duration of stride, stance, and swing phases. Locomotion studies were performed at P24–P25 because the stride parameters are established in their adult form by this age (Clarke and Still, 2001). Each animal attempted 10 trials at speeds incrementing by 10 cm/s between 25 and 95 cm/s; one trial at each speed in which the animal maintained constant position on the treadmill was selected for analysis.

Despite a smaller body size, many Chx10::DTA mice were capable of achieving speeds as fast or faster than wild-type mice (Fig. 2A). Commensurate with smaller body size, Chx10::DTA mice have a shorter stride but take faster steps to run at the same speed as the littermate controls (Fig. 2B,C). As in the control mice, the step duration decreases as Chx10::DTA mice run at faster speeds, and most of the decrease is attributable to shorter stance phase, although the swing phase is unaltered (Fig. 2D). These data indicate that the loss of V2a interneurons in the Chx10::DTA mice does not significantly alter their ability to run at a wide range of speeds. The individual limb step cycle parameters are also preserved in the absence of V2a neurons, and Chx10::DTA mice selectively shorten the stance phase as the speed increases, like their littermate controls. These data indicate that, despite their smaller size, Chx10::DTA mice retain many features of the normal step cycle during treadmill locomotion. However, their gaits are markedly different.

Speed-dependent gait change from left–right alternation to synchrony in Chx10::DTA mice

In the normal adult mouse, left–right coordination is an important characteristic of the gait at all speeds. Left–right coordination is perturbed during fictive locomotion in spinal cords isolated from neonatal Chx10::DTA mice (Crone et al., 2008). We used DigiGait to analyze the relative position of forepaws and hindpaws as Chx10::DTA and wild-type mice ran in place on a treadmill moving at various speeds. As expected, the gait pattern of wild-type mice consists of strict alternation between left and right forelegs and hindlegs at slow (25 cm/s) and fast (85 cm/s) treadmill speeds (Fig. 3) (supplemental Videos A, C, available at

treadmill belt and to calculate the average left hindleg stride length (in centimeters) (**B**) and stride frequency (in steps per second) (**C**) of Chx10::DTA mice and wild-type littermates running at speeds ranging from 25 to 85 cm/s. The value shown is the average of all animals able to attain each treadmill speed (see **A**). **D**, The stance phase duration and swing phase duration were calculated for the left hindleg of Chx10::DTA and wild-type mice running at speeds ranging from 25 to 85 cm/s. The value shown is the average of all animals able to attain each treadmill speed (see **A**). Error bars indicate SEM.

www.jneurosci.org as supplemental material). At slow speed (25 cm/s), the gait pattern of Chx10::DTA mice also shows normal alternation between left and right forelegs and the left and right hindlegs (Fig. 3) (supplemental Video B, available at www.jneurosci.org as supplemental material). However, at high speeds, Chx10::DTA mice show a “galloping” gait in which the left and right forelegs as well as left and right hindlegs move synchronously in the same phase (Fig. 3) (supplemental Video D, available at www.jneurosci.org as supplemental material). At both speeds, wild-type as well as Chx10::DTA mice alternate movements of the forelegs and hindlegs on the same side (Fig. 3) (supplemental video, available at www.jneurosci.org as supplemental material). These results demonstrate a speed-dependent disruption of left–right alternation of the forelegs and hindlegs in Chx10::DTA mice, resulting in the expression of a novel galloping gait in the absence of the V2a interneurons.

To test whether the transition from the trotting to the galloping gait is gradual, we analyzed left and right hindleg coordination at speeds ranging from 25 to 95 cm/s. Phase differences between left and right legs range from 0 to 1.0 in which values close to 0.5 represent alternation (Fig. 4A). Examples of the phase values for hindlegs of a wild-type and a Chx10::DTA mouse during 20 consecutive steps at 25, 45, 65, and 85 cm/s are shown in Figure 4B. The wild-type mouse maintains phase values close to 0.5 (alternation) during treadmill locomotion at all speeds. The phase values close to 0.5 are also maintained by the Chx10::DTA mouse at moderate speeds of 25 and 45 cm/s. However, at a faster speed of 65 cm/s, the Chx10::DTA mouse fails to maintain a constant phase difference as hindlegs move in-phase and out-of-phase on a step-by-step basis. At the fastest speed tested, 85 cm/s, this Chx10::DTA mouse stabilizes its gait with consistent synchronous movement of left and right hindlegs. The summary of data from all mice is shown in circular plots of phase difference (Fig. 4C,D), illustrating the change in gait as speed is increased for Chx10::DTA mice ($n = 11$) (Fig. 4D) but not wild-type ($n = 11$) (Fig. 4C) mice. The phase is indicated by direction on circular plots, whereas the consistency of the phase value (R value) is indicated by distance from the center. At low speeds (25–45 cm/s), all mice show consistent hindleg alternation with no significant difference between the R values of trials from Chx10::DTA and wild-type mice (Mann–Whitney U test, $p > 0.05$; $U = 480$; $n = 33$ wild-type and 31 Chx10::DTA trials). However, at moderate speeds (55–65 cm/s), Chx10::DTA mice show much less consistent alternation than wild-type mice, as indicated by an average R value significantly lower for Chx10::DTA than wild-type mice (Mann–Whitney U test, $p < 0.01$; $U = 53$; $n = 19$ wild-type and 16 Chx10::DTA trials). At fast speeds, a synchronous galloping gait was observed in 2 of 7, 1 of 5, 3 of 4, and 2 of 2 Chx10::DTA mice running at 65, 75, 85, and 95 cm/s, respectively, but never in wild-type mice. These data demonstrate a gradual shift from trotting to irregular gait to galloping gait as Chx10::DTA mice run on a treadmill at increasing speeds.

Figure 3. Chx10::DTA mice gallop at fast treadmill speeds. **A–D**, Gait diagrams represent stance phase (black bars) and swing phase (no bars) of each limb during treadmill locomotion. The trotting gait of wild-type mice features left and right hindleg (LH, RH) alternation and left and right foreleg (LF, RF) alternation at both 25 cm/s (**A**) and 85 cm/s (**B**). Chx10::DTA mice trot at 25 cm/s (**C**) but move their left and right hindlegs synchronously in a galloping gait at 85 cm/s (**D**). **E, F**, The gait diagram of a complete step cycle is shown for a wild-type (**E**) and a Chx10::DTA (**F**) mouse running at 85 cm/s. Stance phase is indicated by a black bar and swing phase by no bar for the left and right hindlegs (LH, RH) and left and right forelegs (LF, RF). Still frames of high-speed digital video recorded from underneath the clear treadmill belt are shown for the time points (1–4) to illustrate paw placement.

We next grouped the trials according to stride frequency (steps per second) to compare hindlimb alternation between wild-type mice and the smaller Chx10::DTA littermates moving their limbs at the same rate (supplemental figure, available at www.jneurosci.org as supplemental material). A rate of 7 steps/s was chosen to divide the wild-type trials into slow (<7 steps/s; $n = 31$ trials) and fast (7–9 steps/s; $n = 32$ trials) groups. Trials from Chx10::DTA mice were likewise grouped into slow (<7 steps/s; $n = 15$) and fast (7–9 steps/s; $n = 29$) groups, as well as an additional group (>9 steps/s; $n = 14$) that is faster than any wild-type trial. Wild-type mice consistently alternate their hindlimbs at both slow and fast stride frequencies. Chx10::DTA mice exhibit consistent alternation at stride frequencies <7 steps/s, less consistent alternation at 7–9 steps/s, and inconsistent alternation or synchronous hindlimb movement at stride frequencies >9 steps/s. Importantly, trials from Chx10::DTA mice exhibit a significantly lower R value than trials from wild-type mice when running at 7–9 steps/s (Mann–Whitney U test, $p < 0.05$; $U = 305$; $n = 32$ wild-type and 29 Chx10::DTA trials) but do not show significantly different R values at stride frequencies <7 steps/s (Mann–Whitney U test, $p > 0.05$; $U = 150$; $n = 31$ wild-type and 15 Chx10::DTA trials). These results confirm a gradual decrease in the ability of Chx10::DTA mice to maintain strict hindlimb alternation with increasing step cycle frequency that is not observed in wild-type mice.

Spinal origin of speed-dependent gait transition in Chx10::DTA mice

We next asked whether the galloping gait, observed in Chx10::DTA mice running on a fast-moving treadmill, results from the absence of local V2a interneurons in the lumbar spinal cord. Previously, we found that spinal cords isolated from neonatal Chx10::DTA mice can be induced to generate a locomotor-like rhythm with NMDA and 5-HT (Crone et al., 2008). We

Figure 4. Speed dependence of left-right hindleg alternation in Chx10::DTA mice. **A**, The left-right (L-R) phase value is calculated by subtracting the time between the start of the left hindleg (LH) stance phase and right hindleg (RH) stance phase and dividing by the duration of the right hindleg step cycle. **B**, The left-right phase was plotted for 20 consecutive steps for a wild-type and a Chx10::DTA mouse running at 25, 45, 65, and 85 cm/s. Left-right phase values near 0.5 represent alternation, and values near 0 or 1 represent synchrony. **C**, **D**, Circular plots representing left-right phase (direction) and consistency of phase (distance from center) in trials from wild-type (**C**) and Chx10::DTA (**D**) mice running at speeds from 25 to 95 cm/s. Each dot represents one trial. One trial per mouse is shown for each treadmill speed, and the number of mice capable of achieving each speed is shown in Figure 2A.

analyzed the regularity of L2 ventral root rhythmic bursting cycle frequency using autocorrelation analysis during fictive locomotion evoked by 6 μ M NMDA and 6 μ M 5-HT. As reported previously (Crone et al., 2008), the Chx10::DTA rhythmic activity was less regular as reflected in the lower value of the autocorrelation coefficient (wild type, 0.72 ± 0.12 , $n = 31$; Chx10::DTA, 0.37 ± 0.19 , $n = 56$; $p < 0.001$). Interestingly, 32 of 56 Chx10::DTA spinal cords generated relatively normal alternating locomotor-like activity, characterized by statistically significant left-right as well as ipsilateral flexor-extensor alternations, although the strength of the alternating cross-correlation values was somewhat weaker than the controls (Fig. 5G). This was especially true at lower cycle frequencies, when the burst frequency was < 0.3 Hz. Figure 5A–C shows a Chx10::DTA spinal cord that generated a relatively slow fictive locomotor pattern comparable with wild-type spinal cords and that shows normal left-right and ipsilateral flexor-extensor

alternation. However, the phase between left-right flexor bursts was not stable in 24 Chx10::DTA spinal cords. In some Chx10::DTA cords, we noted continuous shifting of the left-right phase from alternation to synchronization and back to alternation, whereas the flexor-extensor alternation was well maintained (data not shown). Moreover, in three Chx10::DTA spinal cords with relatively high burst frequencies, the left-right phase showed a predominantly synchronized pattern. Figure 5D–F illustrates an example of a Chx10::DTA spinal cord that generated relatively high-frequency rhythmic activity in the presence of NMDA and 5-HT, with synchronized left-right activity. The synchronized left-right burst pattern is indicated by the positive value in the left-right cross-correlogram (Fig. 5E) and the mean phase vector in the circular plot (Fig. 5F), which points to near 0, whereas the flexor-extensor alternating phase was relatively well retained.

These data suggested that the choice between alternation and synchronous activation of left and right L2 roots depends on the frequency of the motor burst activity induced by NMDA and 5-HT. We tested this hypothesis by determining whether there is a significant relationship between the left-right phase (measured by the value of the cross-correlation coefficient) and the burst frequency. As shown in Figure 5G, linear regression analysis of this relationship showed that, in the wild-type spinal cord, there is no significant relationship between burst frequency and left-right phase ($n = 31$; $r = 0.067$; $p > 0.1$). This confirms that the wild-type cord generates locomotor-like activity with strict left-right alternation at all burst frequencies. In contrast, there was a statistically significant positive relationship between burst frequency and the left-right cross-correlation coefficient in the

Chx10::DTA spinal cords during locomotion induced by 6 μ M NMDA and 6 μ M 5-HT ($n = 56$; $r = 0.57$; $p < 0.001$). As the burst frequency increases, the left-right phase changes from alternating to poor left-right coordination and finally to predominantly synchronized activity. Thus, unlike the wild-type cord, the degree and the sign of left-right coordination varies with fictive locomotor speed, just as was seen during locomotion in the intact mouse. We also analyzed the relationship between burst frequency and ipsilateral flexor-extensor phase in the wild-type and Chx10::DTA spinal cords, but no significant relationship was found in either strain, demonstrating that flexor-extensor phasing is maintained at all frequencies. These results demonstrate that Chx10::DTA spinal cords can generate relatively normal phasing of fictive locomotion at lower burst frequencies, but the left-right phase shifts toward synchronization at high burst frequencies.

Speed-dependent, reversible shift in left–right coordination of motor activity in individual Chx10::DTA spinal cords

To further explore the changes in left–right phasing with frequency in Chx10::DTA cords, we took advantage of the fact that higher concentrations of NMDA and 5-HT can reversibly evoke somewhat higher frequencies of fictive locomotion. The threshold for drug-induced rhythmic activity was tested in spinal cords isolated from wild-type and Chx10::DTA mice. In both mice, 5 μ M NMDA and 5 μ M 5-HT (5:5 for NMDA/5-HT) reliably evoked stable rhythmic ventral root bursting, whereas lower concentrations (2:2 or 4:4) failed to do so (wild type, $n = 8$; Chx10::DTA, $n = 8$). These results suggest that the transmitter threshold for the network to generate rhythmic activity is not significantly altered in Chx10::DTA mice. This allowed us to examine frequency-dependent changes in left–right phasing within a single cord as different concentrations of NMDA and 5-HT were applied. We analyzed the left–right and flexor–extensor phase relationship at different frequencies using cross-correlation and circular plot analysis.

As expected, cords from the wild-type mice continue to show strong left–right and flexor–extensor alternation as frequency is accelerated by increasing the NMDA/5-HT concentration from 6:6 μ M to 10:10 μ M (data not shown). The Chx10::DTA spinal cords also accelerate their burst frequency with elevated NMDA and 5-HT, and this effect is reversible (Fig. 6A). The ipsilateral flexor–extensor relationship is well maintained at the elevated frequency, as analyzed with cross-correlation and circular analysis (Fig. 6B,C). However, the left–right phase is more significantly disturbed when the burst frequency is high. As illustrated in Figure 6B, the value at 0 of the cross-correlogram is less negative at the higher frequency when the burst frequency is higher. Circular analysis shows that the left–right phase loses its significant alternation at the higher frequency; this reverses during return to 6:6 μ M NMDA and 5-HT (Fig. 6B,C). Figure 6D summarizes the changes in left–right cross-correlation coefficient as a function of frequency in four Chx10::DTA cords compared with two wild-type cords. In all four Chx10::DTA cords, the left–right phase becomes less negative and less regular when the burst frequency is elevated by high NMDA and 5-HT, although no such change is seen in the wild-type spinal cords (Fig. 6D, left). In both wild-type and Chx10::DTA spinal cords, the increase of burst frequency has no effect on the ipsilateral flexor–extensor phase relationship (Fig. 6D, right). Our results suggest that a single Chx10::DTA spinal cord can exhibit poorer alternation as the burst frequency increases, consistent with behavioral

Figure 5. Frequency dependence of left–right alternation of motor activity in isolated neonatal spinal cords from Chx10::DTA mice. **A**, Representative Chx10::DTA spinal cord with low cycle frequency in 6:6 μ M NMDA/5-HT. Recordings from IL2, rL2, and rL5 ventral roots showing both left–right (IL2, rL2) and ipsilateral flexor–extensor (rL2, rL5) alternation. Bottom three traces are smoothed and rectified versions of the spike data in top three traces. **B**, Cross-correlogram of the left–right (black trace) and the flexor–extensor (gray trace) activity from the cord in **A**, showing strong alternation in both cases. **C**, Circular plots for the left–right and flexor–extensor activity from **A**, showing alternation in both cases. **D**, Representative recording from a different Chx10::DTA cord in which 6:6 μ M NMDA/5-HT induced fictive locomotion at high cycle frequency, showing synchronous activity of left and right ventral roots. **E**, Cross-correlogram for the left–right (black trace) and the flexor–extensor (gray trace) activity from **D**, showing flexor–extensor alternation but left–right synchrony. **F**, Circular plot for the left–right and flexor–extensor activity from **D**, showing left–right synchrony and flexor–extensor alternation. **G**, Linear regression analysis between the burst frequency and the cross-correlation coefficient for left–right activity from wild-type ($n = 31$) and Chx10::DTA ($n = 56$) spinal cords.

data showing that the Chx10::DTA mice generate different gaits at different speeds.

“Fictive galloping” induced by sacral nerve cord stimulation in isolated Chx10::DTA spinal cord

Although fictive locomotion can be evoked by bath application of NMDA and 5-HT, it is a somewhat artificial method that will depolarize virtually all the neurons in the cord. Increasing the speed with increased levels of transmitters accentuates this problem, so we looked for additional ways to evoke high-speed fictive locomotion in isolated spinal cords. Bouts of fictive locomotion can be evoked by more natural stimuli such as sensory nerve or hindbrain stimulation. We have studied fictive locomotion in wild-type animals evoked by stimu-

Figure 6. Reversible, frequency-dependent changes in left-right coordination of a single Chx10::DTA spinal cord. **A**, Representative recordings from IL2, rL2, and rL5 ventral roots of a Chx10::DTA spinal cord during application of 6:6, 10:10, and return to 6:6 μ M NMDA/5-HT. Bottom three traces are smoothed and rectified versions of the spike data in top three traces. **B**, Cross-correlograms for left-right and flexor-extensor activity during application of 6:6 (trace 1), 10:10 (trace 2), and return to 6:6 μ M NMDA/5-HT (trace 3), showing reduction of left-right alternation at higher frequency with no change in flexor-extensor alternation. **C**, Circular plot for the left-right and flexor-extensor activity from **A**. **D**, Data from multiple experiments performed as described in **A**, showing the relationship between cross-correlation coefficient and burst frequency from wild-type (open triangles; $n = 3$) and Chx10::DTA (filled triangles; $n = 5$) spinal cords. Each connected pair of triangles represents one spinal cord at low and high frequencies.

lation of the sacral end of the spinal cord (Whelan et al., 2000; Gordon and Whelan, 2006; Zhong et al., 2007) and asked whether this method also works in the Chx10::DTA cord. In both wild-type and Chx10::DTA spinal cords, low-frequency

tonic caudal cord stimulation evoked short bouts of relatively high-frequency rhythmic activity from the L2 (flexor-dominated) and L5 (extensor-dominated) ventral roots (Fig. 7A). The average frequency evoked by caudal cord stimulation in both wild-type cords (0.55 ± 0.08 Hz; $n = 7$) and Chx10::DTA cords (0.67 ± 0.15 Hz; $n = 8$) are at the highest end of the range we could achieve with drug stimulation (compare with Fig. 5G). The burst frequency evoked by caudal cord stimulation in Chx10::DTA spinal cords was slightly but significantly higher than the wild-type spinal cords ($p < 0.05$). This is reflected in a plot of burst frequency with step cycle from a typical wild-type and a Chx10::DTA spinal cord (Fig. 7D). Thus, caudal cord stimulation provides an independent way of achieving higher-frequency fictive locomotion in both wild-type and Chx10::DTA spinal cords, allowing us to test whether galloping-like, fictive locomotion can be evoked by synaptic input in the absence of V2a interneurons.

In the wild-type spinal cord, a normal locomotor-like pattern, characterized by both left-right and ipsilateral flexor-extensor alternation, was always evoked by sacral cord stimulation (Fig. 7A). When the data were analyzed by circular plot analyses, the average left-right phase was 0.46 ± 0.07 ($n = 7$), whereas the ipsilateral flexor-extensor phase was 0.49 ± 0.06 ($n = 7$) in the wild-type spinal cords (Fig. 7B,C, white arrows). A very different motor pattern was evoked by caudal cord stimulation in the Chx10::DTA spinal cords (Fig. 7A). The ipsilateral flexor-extensor activity showed normal alternation, with an average phase of 0.55 ± 0.08 ($n = 5$) (Fig. 7C, black arrow). However, the left and right L2 roots showed a strongly synchronous bursting pattern (Fig. 7A) with an average left-right phase of 0.95 ± 0.12 in the Chx10::DTA spinal cords ($n = 8$) (Fig. 7B, black arrow). Using Watson's U^2 test for statistical significance of phase differences (Zar, 2000), the synchronous left-right phase in Chx10::DTA spinal cords was significantly different from the alternating phase in wild-type spinal cords ($U^2_{(7,8)} = 0.345$; $p < 0.005$), but the ipsilateral alternating flexor-extensor phases were not different between the two strains ($U^2_{(7,5)} = 0.134$; $p > 0.1$).

These results show that, during high-speed fictive locomotion evoked by synaptic drive, the Chx10::DTA spinal cord generates synchronous galloping rhythmic activity from the lumbar ventral roots rather than the normal left-right alternation seen in wild-type cords.

Discussion

Although atypical for mice, the switch from alternation to synchronous movement of the left and the right legs at higher speeds is the normal gait pattern for many animals. This study provides direct experimental evidence that interneurons in the spinal cord play an important role in speed-dependent change in gait. Transgenic mice (Chx10::DTA mice) in which V2a interneurons are ablated show normal trotting gait at slow speeds but transition to a galloping gait as speed increases. Although left–right limb coordination is altered in Chx10::DTA mice at fast speed, alternation of forelegs and hindlegs and the relative duration of swing and stance phases for individual limbs is unchanged compared with wild-type mice. The speed-dependent switch from left–right alternation toward synchrony is recapitulated during drug-induced locomotion in spinal cords isolated from neonatal Chx10::DTA mice and at the highest speeds of fictive locomotion evoked by tonic stimulation of the caudal end of the spinal cord. This occurs despite the large differences in locomotor cycle frequency *in vivo* and *in vitro*. These experiments suggest that spinal V2a interneurons are required for maintaining normal left–right alternation but only at fast speeds.

Genetic background and neonatal lethality in the absence of V2a interneurons

To study treadmill locomotion in an intact mouse, it was necessary to rescue the neonatal lethality reported previously for Chx10::DTA mice on a mixed BL6/129 genetic background (Crone et al., 2008). Mouse strain differences are known to impact respiratory stability in the mouse (Han and Strohl, 2000; Han et al., 2001, 2002; Schlenker et al., 2006), and BL6 mice have a predisposition to irregular breathing and apneas (Stettner et al., 2008). We decided to intercross the Chx10::DTA males with ICR females and found increased survival (40%) in Chx10::DTA offspring on this mixed ICR/BL6/129 genetic background. Importantly, survival of Chx10::DTA mice is not attributable to ineffective neuronal ablation, because V2a neurons are nearly absent from both the spinal cord and medulla in both strains of mice. The ablation appears specific for V2a neurons in Chx10::DTA mice produced by both ICR dams and BL6 dams (this study and Crone et al., 2008). When treated neonatally with naloxone, some of the Chx10::DTA mice produced by BL6 dams survive; they show the same locomotor phenotype of normal alternating locomotion at low speeds and synchronized galloping at high speeds as mice produced by ICR dams (data not shown). Furthermore, the phenotype observed during

Figure 7. Synchronous left–right activity in Chx10::DTA spinal cords during fictive locomotion evoked by stimulation of the sacral end of the spinal cord. **A**, Rhythmic locomotor-like activity triggered by caudal cord stimulation in a wild-type (left) and Chx10::DTA (right) spinal cord. The stimulation artifact was removed with a custom-written Spike2 script. **B**, **C**, Circular plots of peak phase relations between left–right (**B**) and ipsilateral flexor–extensor (**C**) bursts in wild-type ($n = 7$) and Chx10::DTA ($n = 8$) spinal cords. The open and filled arrow vectors show the mean phases from all experiments for each type of cord. **D**, Stable differences in burst frequency over time for the wild-type and Chx10::DTA spinal cords in **A**.

transmitter-induced fictive locomotion is identical in isolated neonatal spinal cord preparations from Chx10::DTA mice from ICR and BL6 dams.

V2a interneurons are dispensable for locomotor rhythm generation and for flexor–extensor coordination

In vivo, mature Chx10::DTA mice spontaneously initiate open-field locomotion and locomote appropriately on a treadmill. During treadmill runs, Chx10::DTA mice exhibit regular alternation of swing and stance phase. This finding is consistent with the observations in isolated spinal cord preparations that both electrically and chemically induced locomotor rhythms show normal alternation of ipsilateral flexor (L2) and extensor (L5) ventral roots in both Chx10::DTA and wild-type mice. An important characteristic of locomotion is preferential shortening of the extensor phase with increases in speed (Clarke and Still, 1999; Kriellaars et al., 1994; Yakovenko et al., 2005; Rossignol et al., 2006; Juvin et al., 2007).

Figure 8. Role of V2a interneurons in speed-dependent control of gait. In the lumbar spinal cord, functionally inhibitory commissural interneuron pathways (ICIN) promote alternation of the left and right locomotor CPGs (gray circles), whereas the functionally excitatory commissural interneuron pathways (ECIN) promote synchrony between the left and right sides. In wild-type mice, an ICIN pathway that does not require V2a neurons for activation functions at slow speed to maintain left–right alternation and causes mice to trot. At fast speed, an ICIN pathway that requires excitatory drive from V2a neurons ensures left–right alternation and a trotting gait. Mice lacking V2a neurons trot normally at slow speed because the V2a-independent ICIN is active. However, at fast speed, the V2a-dependent ICIN pathway is not active and the ECIN pathway dominates, causing Chx10::DTA mice to gallop. The relative strength of each pathway is indicated by line width. Excitatory pathways end with arrows, and inhibitory pathways end with crossbars.

This characteristic is preserved in the absence of V2a interneurons. Similarly, the alternation of forelegs and hindlegs on the same side is also preserved in the absence of V2a interneurons. Neonatal (P0) Chx10::DTA mice exhibit spontaneous limb movements. In the isolated neonatal spinal cord, electrical stimulation of the sacral spinal cord (including both afferents and ascending interneurons) reliably initiates fictive locomotion (although with synchronous rather than alternating left–right activity) in neonatal Chx10::DTA spinal cords, as shown previously for wild-type spinal cords (Gordon and Whelan, 2006; Zhong et al., 2007). Thus, locomotion can be initiated *in vivo* and *in vitro* in the absence of V2a interneurons.

V2a interneurons control gait at high speed

Running on a treadmill, as the speed increases, Chx10::DTA mice switch from a trot to a poorly coordinated intermediate state and finally to a gallop, whereas wild-type mice show a trotting gait at all speeds. This variability in left–right limb coordination is consistent with the anatomical finding that excitatory V2a interneurons synapse onto commissural neurons, which coordinate activity in the left and right sides of the spinal cord (Crone et al., 2008). The speed dependence of V2a neuron function is consistent with the role of the CiD interneurons, which express the Chx10 homolog *alx* in the zebrafish; a subset of these interneurons is selectively activated only at high swim speeds (Kimura et al., 2006). This speed-dependent role of V2a interneurons in left–right coordination is observed even in the isolated spinal cord. How might V2a interneurons integrate the frequency of locomotor activity and coordination of the left–right CPGs?

Figure 8 presents a simple model for the role of the V2a interneurons in speed-dependent regulation of gait. The co-

ordination of left–right motor activity is mediated by commissural interneuron (CIN) systems that are functionally inhibitory or excitatory (Cohen and Harris-Warrick, 1984; Cazalets et al., 1994; Hagevik and McClellan, 1994; Cowley and Schmidt, 1995; Bracci et al., 1996; Butt and Kiehn, 2003; Hinckley et al., 2005; Quinlan and Kiehn, 2007). The relative activity of the functionally excitatory and inhibitory CIN pathways determines whether the hindlimbs alternate to produce trotting or are active synchronously to produce galloping (Cohen and Harris-Warrick, 1984; Kullander et al., 2003). Synchronous, rather than uncoupled, left–right leg movements in Chx10::DTA mice at the highest speeds indicate that the functionally excitatory CIN system is intact in mice lacking V2a neurons, consistent with previous studies showing synchronous left–right activity in the disinhibited neonatal Chx10::DTA spinal cord (Crone et al., 2008). Our previous study provided anatomical evidence that V2a neurons provide excitatory drive to the functionally inhibitory CIN system, including the V0 class of CINs. V0 interneurons are necessary for maintaining left–right alternation in isolated spinal

cord preparations (Lanuza et al., 2004). One plausible mechanism underlying the speed dependence of V2a interneuron function is that the V2a–V0 system is the predominant regulator of left–right alternation at fast speeds but is either silent or redundant with another unidentified functionally inhibitory CIN pathway at slow speeds (Fig. 8). The ability of Chx10::DTA mice to alternate left and right legs while running slowly indicates that other CIN systems might be sufficient to maintain alternation at these speeds. The alternative system might also include V0 interneurons, which would then receive excitatory drive from unidentified interneurons other than V2a interneurons. These unidentified interneurons must reside within the spinal cord, because isolated cords from Chx10::DTA mice exhibit normal left–right alternation at slow speed. An alternative possibility is that the commissural excitatory pathway becomes stronger at higher speeds so that, in the absence of the V2a–V0 inhibitory system, the balance of crossed excitation and inhibition is shifted to excitation, resulting in left–right synchrony.

It is not yet clear whether the V2a–V0 system is normally inactive at low speeds or whether it is merely unnecessary because of the presence of an additional alternating system at slow speeds. In zebrafish and *Xenopus* embryos, the speed of locomotion is correlated with selective activation and inactivation of different subsets of spinal interneurons, thus changing the neuronal composition of the locomotor CPG as a function of cycle frequency (Sillar and Roberts, 1993; Ritter et al., 2001; Kimura et al., 2006; McLean et al., 2008). Our results are consistent with the hypothesis that the CPG neuronal composition changes with speed in mammals as well. Based on the genetic similarities among vertebrates in specifying the principle neural cell types (V0–V3) in the developing ventral spi-

nal cord (Goulding and Pfaff, 2005; Lewis, 2006) and similar properties of En1 neurons (Higashijima et al., 2004) and of Chx10/Alx neurons (Kimura et al., 2006) in mammals and zebrafish, it would not be surprising that similar strategies might be used for their recruitment during locomotor behavior. We propose that, in the mouse, the V2a–V0 system is the predominant circuit driving left–right alternation at fast speeds, whereas an alternative circuit functions at slow speeds (Fig. 8). As shown in Figure 8, one way to do this is for the V2a–V0 system to be weak or silent at low speeds but more active at high speeds, whereas the alternative functionally inhibitory system is active at low speeds and weak or silent at high speeds. Thus, in the absence of the V2a interneurons in Chx10::DTA mice, the functionally excitatory crossed pathway will dominate at high speeds, causing synchronous left–right limb coordination and a galloping gait.

In conclusion, we find that left–right alternation is progressively lost and eventually replaced by synchronous galloping as Chx10::DTA mice run faster. This speed-dependent switch in gait is likely attributable to the loss of spinal V2a interneurons, because it can be recapitulated during fictive locomotion in the isolated Chx10::DTA spinal cord. These data demonstrate that V2a interneurons in the lower spinal cord are responsible for maintaining left–right alternation but only at higher speeds of locomotion. Thus, in mice, the requirement for V2a neurons to maintain left–right limb alternation is dependent on locomotor speed.

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