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# Identification of Minimal Neuronal Networks Involved in Flexor-Extensor Alternation in the Mammalian Spinal Cord

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## SUMMARY

**Neural networks in the spinal cord control two basic features of locomotor movements: rhythm generation and pattern generation. Rhythm generation is generally considered to be dependent on glutamatergic excitatory neurons. Pattern generation involves neural circuits controlling left-right alternation, which has been described in great detail, and flexor-extensor alternation, which remains poorly understood. Here, we use a mouse model in which glutamatergic neurotransmission has been ablated in the locomotor region of the spinal cord. The isolated in vitro spinal cord from these mice produces locomotor-like activity—when stimulated with neuroactive substances—with prominent flexor-extensor alternation. Under these conditions, unlike in control mice, networks of inhibitory interneurons generate the rhythmic activity. In the absence of glutamatergic synaptic transmission, the flexor-extensor alternation appears to be generated by Ia inhibitory interneurons, which mediate reciprocal inhibition from muscle proprioceptors to antagonist motor neurons. Our study defines a minimal inhibitory network that is needed to produce flexor-extensor alternation during locomotion.**

## INTRODUCTION

Locomotion is a complex, rhythmic motor behavior that involves coordinated activation of a large group of muscles. In all vertebrates, the generation of locomotion is largely determined by neural networks located in the spinal cord. Spinal locomotor networks need to serve two basic functions: rhythm generation and pattern generation. Spinal glutamatergic excitatory neurons are generally considered to be indispensable for rhythm generation in all vertebrate locomotor networks (Grillner, 2006; Kiehn, 2006). Thus, a blockade of intrinsic network ionotropic glutamatergic receptors results in attenuation or disruption of locomotor activity (Talpalar and Kiehn, 2010; Whelan et al., 2000). The pattern generation involves left-right alternation and, in limbed animals with multiple joints, flexor-extensor alternation. The neural circuits in mammals underlying left-right alternation have been determined in great detail (Jankowska, 2008; Kiehn, 2011; Quinlan and Kiehn, 2007). The locomotor network generating flexor-extensor alternation appears to be generated by reciprocally connected flexor and extensor modules. However, the nature of the interneuron groups involved in generating flexor-extensor alternation remains poorly understood.

Alternation between flexor and extensor muscles within a limb or around joints depends on activity in ipsilaterally projecting inhibitory networks. Thus, alternation between flexors and extensors persists in the hemicord (Kjaerulff and Kiehn, 1997; Whelan et al., 2000), and blocking fast GABAergic/glycinergic inhibition results in flexors and extensors being activated in synchrony (Cowley and Schmidt, 1995; Hinckley et al., 2005). Ia inhibitory interneurons that are activated by group Ia afferents originating in agonist muscle spindles and that monosynaptically inhibit motor neurons innervating the antagonist muscle have been implicated in this coordination. The connectivity pattern of these reciprocal Ia interneurons (rIa-INs) was first defined in the cat spinal cord (Hultborn et al., 1976; Hultborn et al., 1971a, 1971b), and parts of this connectivity pattern have been described in newborn mice (Wang et al., 2008). rIa-INs are rhythmically active during locomotion (Geertsen et al., 2011; Pratt and Jordan, 1987). In an attempt to associate the rIa-INs with flexor-extensor alternation, the V1 population marked by the transcription factor En1 has been genetically ablated (Gosgnach et al., 2006). En1-expressing neurons are all inhibitory and ipsilaterally projecting and give rise to rIa-INs and inhibitory Renshaw cells, in addition to unidentified inhibitory neurons (Gosgnach et al., 2006; Sapir et al., 2004). However, flexor-extensor alternation and rhythmic inhibition are preserved in the absence of V1 interneurons, either because rIa-INs are not involved in the flexor-extensor alternation and rhythmic inhibition or because Ia-INs, in addition to being derived from the V1 interneuron population, are derived from other developmentally determined inhibitory interneurons (Wang et al., 2008; Goulding, 2009). Eliminating V1 interneurons alone may therefore be insufficient to perturb the flexor-extensor alternation (Grillner and Jessell, 2009; Goulding, 2009; Kiehn, 2011; Stepien and Arber, 2008).

Here we take a different approach to define the networks responsible for flexor-extensor coordination, namely elimination of excitatory synaptic transmission. These experiments were prompted by the recent observation that locomotor-like activity can be induced by drugs in the isolated spinal cord of the perinatal mice when the vesicular glutamate transporter 2, Vglut2, was genetically eliminated from all neurons in the nervous system (Wallén-Mackenzie et al., 2006). Of the three known vesicular glutamate transporters, only Vglut2 is expressed in neurons of the ventral spinal cord where the locomotor network is localized (Borgius et al., 2010). Eliminating Vglut2 should therefore physiologically inactivate all glutamatergic neurons in the locomotor network. We have used a similar mouse model in which the gene encoding Vglut2 is inactivated, and our results show that the Vglut2-mediated glutamatergic neurotransmission is completely blocked in the ventral spinal cord with no detectable compensatory regulation of other excitatory or inhibitory vesicular transporters. Drug-induced locomotor-like activity can be generated in the Vglut2 knockout mice by networks of inhibitory neurons. We provide compelling evidence that the core of this inhibitory network is composed of mutually inhibitory rIa-INs that can coordinate flexor-extensor alternation and that, in the absence of excitatory neurotransmission, can also generate the rhythm. Our study shows that by genetically inactivating excitatory neurons from the locomotor network, it is possible to define essential elements of the pattern-generation circuits in the mammalian spinal cord.

## RESULTS

### Vglut2 Knockout Mice

Complete inactivation of the gene encoding Vglut2 (*Slc17a6*) was achieved by crossing mice that were heterozygous for this allele (Figure S1 available online). The resulting offspring included Vglut2 null mice (referred to as Vglut2 knockouts, Vglut2-KO), mice heterozygous for the gene, and wild-type mice. The Vglut2-KO mice lacked Vglut2 protein in the brain and spinal cord (Figure S1C). All experiments were done on E18.5 embryos because Vglut2-KO mice do not breathe. Heterozygotes and wild-type E18.5 embryos were indistinguishable in their behavior and will be referred to as controls when compared to Vglut2-KO animals.

### Spontaneous Glutamate Release Is Severely Suppressed in the Spinal Cord in Vglut2-KO Mice

To evaluate the consequences of disrupting Vglut2 expression on glutamatergic synaptic transmission in the spinal cord, we first recorded spontaneous synaptic activity in motor neurons (MNs) and neurons in the ventral spinal cord in Vglut2-KO E18.5 mice and compared it with the activity observed in control mice.

Whole-cell recordings from MNs in control animals showed frequent spontaneous barrages of synaptic events, including excitatory postsynaptic events that occurred in long-lasting bursts separated by epochs containing relatively fewer postsynaptic events (Figure 1A). The frequencies of excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) were 11.7 ± 2 Hz and 3.5 ± 1.1 Hz (n = 5), respectively. Both spontaneous EPSCs and IPSCs were blocked by glutamate receptor (GluR) antagonists (Figure 1A, bottom; n = 2), suggesting that excitatory premotor neurons are spontaneously active and provide inputs to both MNs and inhibitory premotor neurons in control mice. In contrast, MNs recorded in Vglut2-KO mice showed no spontaneous barrages of synaptic potentials and few, infrequent EPSCs (1.1 ± 0.6 Hz; n = 4) and IPSCs (1.5 ± 0.5 Hz; n = 4). GluR antagonists blocked both EPSCs and IPSCs (Figure 1B; n = 2).

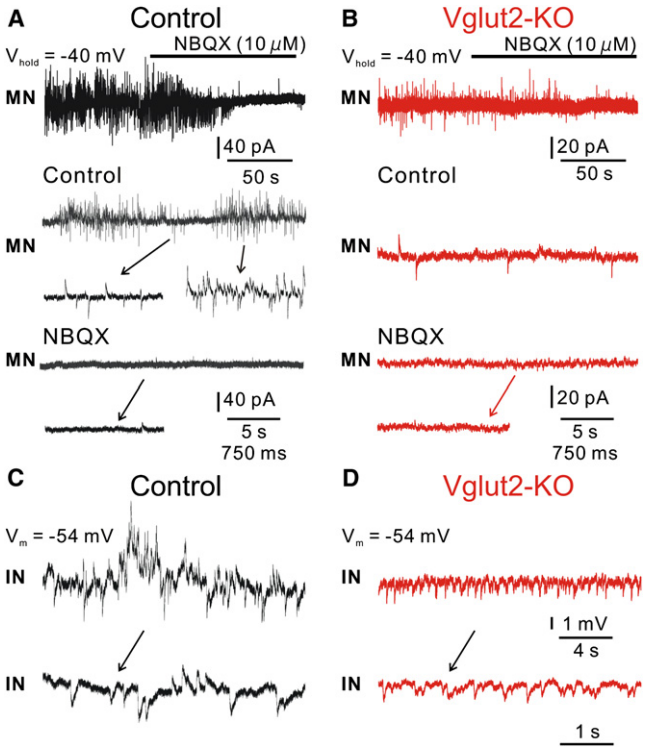


**Figure 1. Spontaneous Glutamatergic Transmission Is Strongly Reduced in Vglut2-KO Mice**

(A and B) Intracellular voltage-clamp recordings (holding potential, $V_{hold}$ = −40 mV) from motor neurons (MNs) in control (A) and Vglut2-KO (B) mice showing spontaneous bursts of EPSCs and IPSCs before and after blockade of glutamatergic transmission with NBQX (10 μM). Action-potential generation was blocked in the recorded MN by including QX-314 in the pipette. Middle and lower traces show expansions of the upper traces. Note that the spontaneous synaptic activity is strongly reduced in Vglut2-KO mice.

(C and D) Intracellular current-clamp recordings from unidentified neurons (INs) located in the lateral ventral region of the lumbar spinal cord of a control (C) and a Vglut2-KO (D) mouse. The membrane potential was kept at −54 mV. Controls showed bursts of EPSPs and IPSPs. Vglut2-KO mice displayed almost exclusively bursts of IPSPs.

Similarly, recordings from unidentified spinal neurons located outside the motor nucleus showed more frequent spontaneous synaptic potentials in control mice (Figure 1C; EPSP frequency 1–5 Hz, IPSP frequency 1–5 Hz, n = 10) than in Vglut2-KO mice (Figure 1D; EPSP frequency 0–0.5 Hz, IPSP frequency 1–5 Hz, n = 4).

These data show that there is a substantial reduction in spontaneous glutamatergic neurotransmission in the spinal cords of Vglut2-KO mice, as compared to controls. The remaining spontaneous glutamate release may be from Vglut1- or Vglut3-positive terminals. There are few Vglut3-positive terminals in

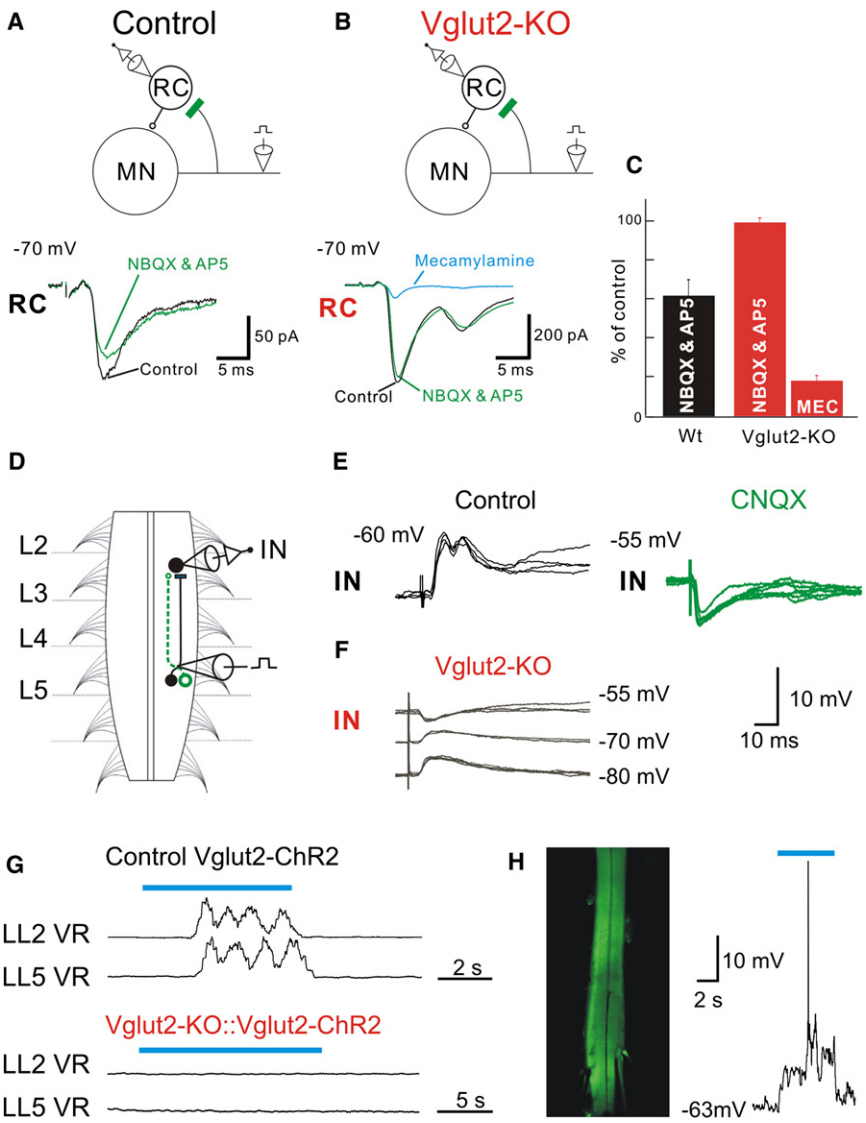


**Figure 2. Evoked Vglut2-Dependent Synaptic Release Is Absent in Vglut2-KO Mice**

(A and B) Intracellular voltage-clamp recordings from Renshaw cells (RCs) in control (A) and Vglut2-KO (B) mice during antidromic stimulation of the motor neuron axons. Spike generation in the RCs was blocked by including QX-314 in the pipette solution. The RCs were held at −70 mV. In control mice, the EPSC was composed of a glutamatergic non-NMDA/NBQX-sensitive (10 μM) component mixed with a cholinergic component. In Vglut2-KO mice, the glutamatergic EPSC component was absent, while the remaining cholinergic EPSC component appeared normal. The cholinergic component was strongly reduced by 50 μM mecamylamine (MEC).

(C) Quantification of the effect of blocking the antidromically elicited EPSCs in RCs by NBQX and AP5 in control and Vglut2-KO mice and by MEC in Vglut2-KO mice. Error bars represent ± SEM.

(D–F) Whole-cell current-clamp recordings of synaptic events elicited by electrical stimulation of the ipsilateral L4-L5 segments in ventrolaterally located neurons in the L2 lumbar segment (D, diagram) in control (E) and Vglut2-KO (F) mice. In control mice, stimulation evoked a compound EPSP-IPSP (E). Blockade of the glutamatergic EPSP by CNQX (20 μM) unveils a remnant IPSP. Stimulation in Vglut2-KO mice never evoked EPSPs but only evoked IPSPs (F).

(G and H) Animals expressing Channelrhodopsin-2 (ChR2) in Vglut2-positive cells display rhythmic activity in response to light stimulation of reticulospinal neurons in the hindbrain (G, upper trace). This effect was completely abolished in the Vglut2-KO mice (G, lower trace), although the spinal cord showed expression of ChR2-YFP (H, left) and the YFP-positive cells could still be activated by light (H, right).

See also Figure S2.

the spinal cord at E18.5, whereas Vglut1 is found in proprioceptive primary afferent terminals in the ventral spinal cord (Hughes et al., 2004; Pecho-Vrieseling et al., 2009), suggesting that some EPSPs are due to spontaneous glutamate release from proprioceptive afferent terminals. The other source of Vglut1-positive terminals is from descending, mainly corticospinal, tracts that have not yet invaded the lumbar spinal cord at this developmental age (Gianino et al., 1999). Alternatively, glutamate may still be released from terminals normally containing Vglut2, despite the lack of protein.

### Action Potential-Evoked Glutamate Release Is Absent in Vglut2 Terminals

To test whether glutamate was still released from terminals containing Vglut2 in Vglut2-KO mice, we examined stimulus-evoked responses in a number of neural pathways that are known to contain Vglut2. These pathways include MN-to-Renshaw cell (RC) (Nishimaru et al., 2005) and intraspinal connections.

Similar to what was previously seen during intracellular recordings from RCs in newborn mice (Mentis et al., 2005; Nishimaru et al., 2005), antidromic activation of motor neuron axons in control E18.5 littermates generated a compound EPSC (amplitude: −182 ± −62 pA [± standard error of the mean (SEM)] at −70 mV; range: −87 to −300 pA; latency from stimulus to onset: 4.1 ± 0.3 ms; n = 3) involving both cholinergic (d-tubocurarine/mecamylamine-sensitive) and glutamatergic (NBQX/AP5-sensitive) fractions (Figures 2A and 2C; n = 3). Recordings from RCs in Vglut2-KO mice (Figure 2B) showed a conserved cholinergic fraction (amplitude: −236 ± −72 pA at −70 mV; range: −26 to −400 pA; n = 5) but a total absence of the glutamatergic component (Figures 2B and 2C; latency from stimulus to onset: 4.2 ± 0.1 ms; n = 7). The cholinergic EPSC was reduced to 17% ± 3% (n = 3) of the control amplitude in Vglut2-KO mice by mecamylamine (Figures 2B and 2C), similar to what was seen in wild-type animals when the glutamatergic component was blocked (Nishimaru et al., 2005). Moreover, in control mice, recordings

from MNs displayed antidromically induced compound EPSCs involving cholinergic and glutamatergic fractions, similar to what has been described in wild-type mice (Nishimaru et al., 2005). In contrast, this glutamatergic fraction was absent in recordings from motor neurons in Vglut2-KO mice (n = 2; data not shown).

During intracellular recordings from ventrally located neurons in the rostral lumbar cord (L2) of control mice (Figure 2E, left), stimulation of ipsilateral caudal segments elicited both EPSPs and IPSPs in the recorded neurons. The IPSPs were often obscured by EPSPs and were only revealed after blocking the EPSPs (Figure 2E, right). Stimulus-evoked EPSPs were seen in all ventrally recorded neurons in control mice (n = 22). Similar recordings in Vglut2-KO mice showed conserved stimulus-evoked IPSPs but a total lack of stimulus-evoked EPSPs (Figure 2F; n = 7).

Stimulation of the ventral funiculus in the caudal spinal cord (L6-S1) leads to glutamatergic excitation of motor neurons in more rostral segments on the same side (e.g., L2 and L5) (Figure S2B). This excitation was absent in Vglut2-KO mice (Figure S2C; n = 3).

We further tested whether release was blocked from Vglut2-positive neurons that have fibers projecting toward or into the spinal cord. For this we obtained crosses that were Vglut2 deficient and carried a BAC transgene expressing channelrhodopsin2-YFP in cells that normally express Vglut2 (Hägglund et al., 2010). In Vglut2-proficient BAC-Vglut2-ChR2-YFP mice, lumbar locomotor-like activity can be induced by blue light stimulation of Vglut2-expressing reticulospinal neurons in the brainstem or propriospinal neurons in the upper cervical spinal cord. Similar light stimulation in Vglut2-KO::BAC-Vglut2-ChR2-YFP mice was unable to evoke a response in the lumbar spinal cord (Figure 2G; n = 5/5), although YFP-positive cells were indeed activated by light (Figure 2H). Direct light stimulation of the spinal cord that effectively evoked locomotor-like activity in BAC-Vglut2-ChR2-YFP Vglut2-proficient mice (Hägglund et al., 2010) was also unable to induce rhythmic activity in Vglut2-KO::BAC-Vglut2-ChR2-YFP (data not shown).

These results show that the Vglut2-KO mice display a specific loss of the stimulus-evoked Vglut2-mediated glutamate release.

## There Is No Compensatory Increase of Other Vesicular Transporters in the Vglut2-KO Mice

To test whether the elimination of Vglut2 leads to compensatory changes in other vesicular transporters, we compared the quantitative protein expression of Vglut2, the two other known vesicular glutamate transporters, Vglut1 and Vglut3, the glutamate/aspartate transporter Sialin, the vesicular acetylcholine transporter VAChT, the vesicular GABA/glycine transporter VIAAT in Vglut2-KO, and control mice. In the spinal cord, the Vglut2 protein was completely absent in Vglut2-KO mice (n = 5), whereas the protein levels for Vglut1, VAChT, and VIAAT were similar in Vglut2-KO mice compared to controls (Figure 3A; $p > 0.05$). The concentration of Vglut3 and Sialin protein in the spinal cord was very low, and the protein levels of these transporters were therefore compared in the brains of Vglut2-KO and control mice. There was no difference in the expression levels. Similarly, when using real-time PCR on lumbar spinal cord tissue of Vglut2-KO and control mice, we found no difference in expression levels of these transporters (data not shown).

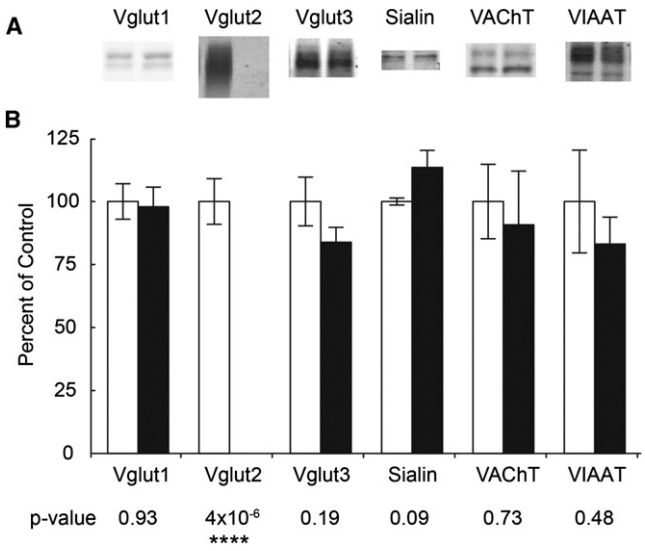


**Figure 3. Absence of Vglut2 Does Not Lead to Compensation by Other Vesicular Excitatory Transporters**

(A) Western blots of Vglut1, Vglut2, VAChT, and VIAAT from spinal cord and Vglut3 and Sialin from brain. Samples display transporter blots in the same animal for control (left) and Vglut2-KO (right) mice.

(B) Quantification of western blots normalized to actin. Percent of change in Vglut2-KO compared to wild-type mice (mean ± SEM; n = 5).

These observations suggest that there is no major compensatory regulation of neurotransmitter vesicular transporters to replace Vglut2 in Vglut2-deficient neurons.

## Neural-Induced Locomotor-Like Activity Is Abolished in the Vglut2-KO Mice

Spinal locomotor activity in mammals can be initiated by stimulation of peripheral sensory afferents (Lev-Tov et al., 2000; Whelan et al., 2000) and by stimulation of glutamatergic neurons located in the lower hindbrain (Hägglund et al., 2010; Jordan et al., 2008). To evaluate the locomotor capability of the Vglut2-KO mice, we first determined whether these animals were able to produce locomotor-like activity in response to electrical stimulation of these neural pathways.

Prolonged low frequency stimulation (0.5–1 Hz) of the midline in the caudal hindbrain or the ventral midline of the rostral (C1-C4) spinal cord was able to elicit a stable locomotor-like activity in spinal cords of E18.5 control mice (n = 12/12), displaying left-right alternation (RL2-LL2 or RL5-LL5) and alternation between the flexor-dominated L2 and extensor-dominated L5 roots on either side of the cord (RL2-RL5 or LL2-LL5), comparable to that elicited in newborn wild-type mice (Figure 4A, left; Talpalar and Kiehn, 2010; Zaporozhets et al., 2004). In contrast, in Vglut2-KO littermates the same stimuli did not evoke any rhythmic activity in the lumbar spinal cord (Figure 4A, right; n = 9/9). Tonic activity that was insensitive to blockade of ionotropic receptors (data not shown) often accompanied the stimulation (Figure 4A, right), possibly as a consequence of stimulating descending Vglut2-negative fibers (e.g., serotoninergic fibers; see Jordan et al., 2008). Increasing the frequency (>1 Hz), the stimulus intensity, or the duration of the stimulus pulses (from 5 to 15 ms) above

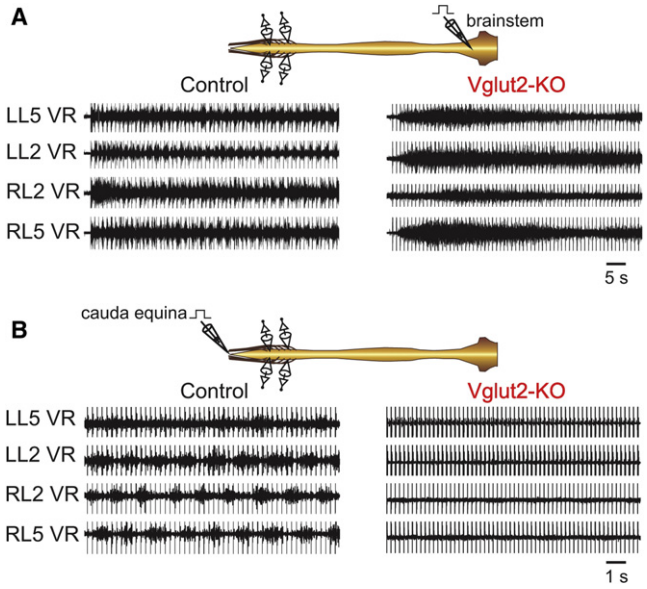


**Figure 4. Vglut2-Dependent Glutamatergic Inputs Are Necessary for Evoking Brainstem- and Afferent-Evoked Locomotor-Like Activity**

(A) Brainstem stimulation elicited regular locomotor-like activity in the lumbar spinal cord in control (left) but not in Vglut2-KO (right) mice. The stimulation did produce tonic activity in Vglut2-KO mice.

(B) Cauda equina or dorsal root stimulation elicited locomotor-like activity in control (left) but not in Vglut2-KO (right) mice.

See also Figure S3.

those able to evoke locomotor-like activity in controls did not evoke rhythmic activity in Vglut2-KO mice (Figure S3; n = 3/3).

Prolonged stimulation of lumbar dorsal roots (L1-L5; n = 4) or stimulation of the cauda equina (n = 6) at low frequencies (0.5–2 Hz) also initiated bouts of locomotor-like activity in control mice (Figure 4B, left), similar to what is seen in wild-type neonatal mice (Marchetti et al., 2001; Whelan et al., 2000). However, in Vglut2-KO littermates (n = 4), dorsal root or cauda equina stimulation was unable to produce locomotor-like activity, although the stimulation often produced tonic activity (Figure 4B, right), possibly by activation of glutamatergic Vglut1-dependent pathways. Even when the general excitability of neurons in the spinal cord was increased with small doses of 5-HT (5 μM), it was impossible to evoke rhythmic activity with afferent stimulation in Vglut2-KO mice (n = 2/2; data not shown).

These results show that glutamate release by Vglut2-expressing neurons is essential for evoking locomotor-like activity.

## Neuroactive Drugs Evoke Rhythmic Activity in Vglut2-KO Mice that Resembles Normal Locomotor-Like Activity

We next examined the ability of neuroactive drugs to induce locomotor-like activity in the isolated spinal cords from mutants. The study by Wallén-Mackenzie et al. (2006) reported that locomotor-like activity could be elicited by bath application of NMDA and 5-HT in isolated spinal cords from Vglut2-KO mice. Bath application of equivalent concentrations of NMDA (4–7 μM) and 5-HT (5–30 μM), as used in Wallén-Mackenzie et al. (2006), induced regular locomotor-like activity in E18.5 control mice (Figure 5B; n = 22). This activity was characterized by alternation (phase values around 0.5) between ipsilateral flexor-related (L2) and extensor-related (L5) ventral roots and left-right alternation (phase values around 0.5) at the segmental level (Figures 5B and 5C, upper left), similar to what is recorded in newborn wild-type mice. In E18.5 Vglut2-KO mice, the same concentrations of NMDA and 5-HT did not evoke rhythmic activity but rather induced tonic activity in the ventral roots (n = 5). However, a locomotor-like rhythm could be initiated in Vglut2-KO mice when a large dose of dopamine (DA; 50 μM) was added to the NMDA/5-HT cocktail (Talpalar and Kiehn, 2010; Whelan et al., 2000; Zhang et al., 1996), often after repeated cycles of wash-in and wash-out trials. Compared with controls, this activity was similar in coordination, although it displayed a higher incidence of abnormal coordination characterized by disrupted left-right or flexor-extensor alternation (Figures 5B and 5C). Higher concentrations of NMDA (10–26 μM) added along with high concentrations of 5-HT (8–20 μM) and DA (50 μM) resulted in higher probability for stable locomotor-like activity, with shorter induction time and higher incidence of normal-like coordination in Vglut2-KO mice (n = 20). Noticeably, 5-HT alone or 5-HT and DA in combination were unable to induce coordinated rhythmic activity in Vglut2-KO mice when applied in the absence of NMDA, regardless of concentration.

Rhythmic alternation between L2 and L5 ventral roots was also seen in hemicords of Vglut2-KO mice (n = 4; data not shown).

In wild-type animals, non-NMDA receptor activation provides a strong excitatory drive in the locomotor network (Talpalar and Kiehn, 2010). However, the non-NMDA receptor agonists kainate (0.5–8 μM) or ATPA (1–2 μM) applied in combination with 5-HT and DA were unable to evoke rhythmic activity in Vglut2-KO mice (n = 2).

In wild-type newborn mice, the gradual increase in NMDA concentration on a background of constant concentrations of 5-HT or 5-HT and DA results in a progressive increase in the locomotor frequency (Talpalar and Kiehn, 2010). The obtainable frequency range is 0.2–1.4 Hz. A similar range of frequencies was seen in E18.5 control mice (n = 4), when NMDA was applied together with 5-HT and DA (Figure 5D, black circles) or without DA (Figure 5E, black circles). However, the frequency curves in Vglut2-KO mice (n = 4) showed slopes that were less steep and displaced to the right compared to control mice (Figures 5D and 5E, red squares). The maximal frequencies obtained in Vglut2-KO mice were around 0.4 Hz.

These experiments show that coordinated locomotor-like activity can indeed be induced in Vglut2-KO mice, although with a somewhat more irregular pattern in some cases and more restricted frequency range than in control mice. The rhythm and pattern generation in Vglut2-KO mice are dependent on NMDA receptor activation. The spinal network in Vglut2-KO mice therefore seems to be reduced to a network that functions independently of intrinsic neuron-to-neuron glutamate receptor activation.

## Acute Vglut2-KO Mice Have Similar Locomotor Phenotype as Chronic Vglut2-KO Mice

To further substantiate the presence of locomotor capability observed in the chronic Vglut2-KO mice, we tested whether

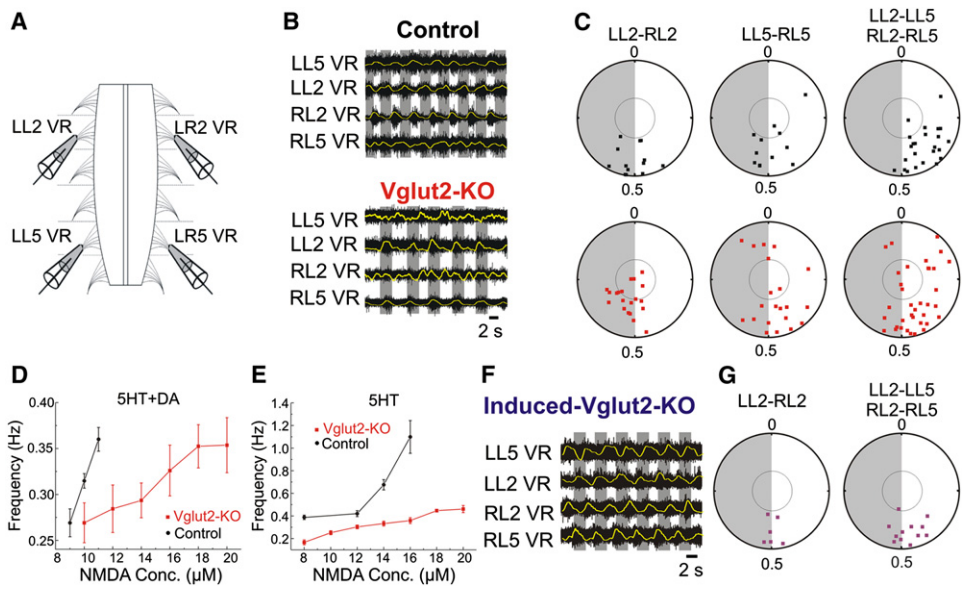


**Figure 5. Neuroactive Drugs Are Able to Evoke Rhythmic Locomotor-Like Activity in Vglut2-KO Mice**

(A) Experimental setup.

(B) Ventral-root recordings of drug-induced rhythmic activity generated in control (upper traces) and Vglut2-KO (lower traces) mice.

(C) Circular plots of left-right (LR2-RL2; LL5-RL5) and flexor-extensor (LL2-LL5; RL2-RL5) coordination in control (upper plots) and Vglut2-KO (lower plots) mice. Each point is the summed vector of 25 cycles in one animal. The inner circle indicates significance at $p < 0.05$.

(D and E) Steady-state frequency of locomotor-like activity as a function of NMDA concentration on a constant background of 5-HT and DA (8 μM + 50 μM) or 5-HT (8 μM) in control (black circles) and Vglut2-KO (red squares) mice. The maximal frequency obtained in Vglut2-KO was lower than in control mice. Error bars represent ± SD.

(F) Drug-induced rhythmic activity generated in tamoxifen-induced Vglut2-KO mice.

(G) Circular plots of left-right and flexor-extensor coordination in induced Vglut2-KO mice.

the basic features of this locomotor phenotype could be reproduced in mice in which Vglut2 was eliminated more acutely and later in development. For this we generated crosses of $Vglut2^{lox/lox}$ and $ROSA26Cre\text{-}ER^{TM}::Vglut2^{+/lox}$ mice in which Cre activity is inducible by tamoxifen. Tamoxifen was given in one dose at E16.5. In tissue harvested at E18.5, this treatment led to an average reduction of the content of Vglut2 protein by 80%–90% (83% ± 4%; mean ± SEM; n = 6) in mice with double-floxed genes and Cre ("induced Vglut2-KO").

The isolated spinal cord from induced Vglut2-KO showed a similar locomotor phenotype as the chronic Vglut2-KO mice: (1) it was impossible to evoke locomotor-like activity by brainstem or afferent stimulation (n = 6/6; data not shown), (2) only combinations of neuroactive substances containing NMDA and 5-HT (with or without DA) could induce stable locomotor-like activity (Figures 5F and 5G), and (3) the locomotor-like activity had a high threshold for induction (average 10 μM NMDA on a constant high 5-HT concentration) and low frequency (<0.4 Hz; n = 6). The locomotor frequency/NMDA concentration curve in these animals was indistinguishable from that of chronic Vglut2-KO mice (n = 6; data not shown).

These experiments show that when the Vglut2 protein levels were reduced in late developmental stages, we obtained a similar locomotor phenotype as seen in the chronic Vglut2-KO mice.

## Network Coordination in Vglut2-KO Mice Is Perturbed by Blocking $GABA_A$ and Glycinergic Neurotransmission

During locomotor-like activity elicited by neurotransmitter agonists in newborn wild-type mice, elimination of the inhibition mediated by glycine and $GABA_A$ receptors leads to abolishment of left-right and flexor-extensor alternation and to occurrence of a rhythmic low-frequency activity characterized by synchronous activation of all lumbar ventral roots (Cowley and Schmidt, 1995; Hinckley et al., 2005). This phenomenon was reproduced in E18.5 control mice by the combined application of the $GABA_A$ receptor blocker picrotoxin (PTX, 10–30 μM) and the glycine receptor blocker strychnine (Strych, 0.3–0.5 μM; n = 5) (Figure 6A: left, control; right, PTX plus Strych). These effects are seen as progressive changes in the phase values of ventral root bursts that are shifted from left-right and flexor-extensor alternation (phase values around 0.5) to left-right and flexor-extensor synchrony (phase values around 0) (Figure 6A1). The circular plots summarize the normal left-right and flexor-extensor alternation (black squares) and their change into synchronization after total blockade of inhibition (red circles) in five independent experiments using control E18.5 embryos (Figure 6A2).

A prominent difference in this pattern was seen in Vglut2-KO mice. Here, during NMDA/5-HT/DA induced rhythmic activity, the combined application of the same doses of PTX and strychnine initially increased the frequency of the activity, then slowed

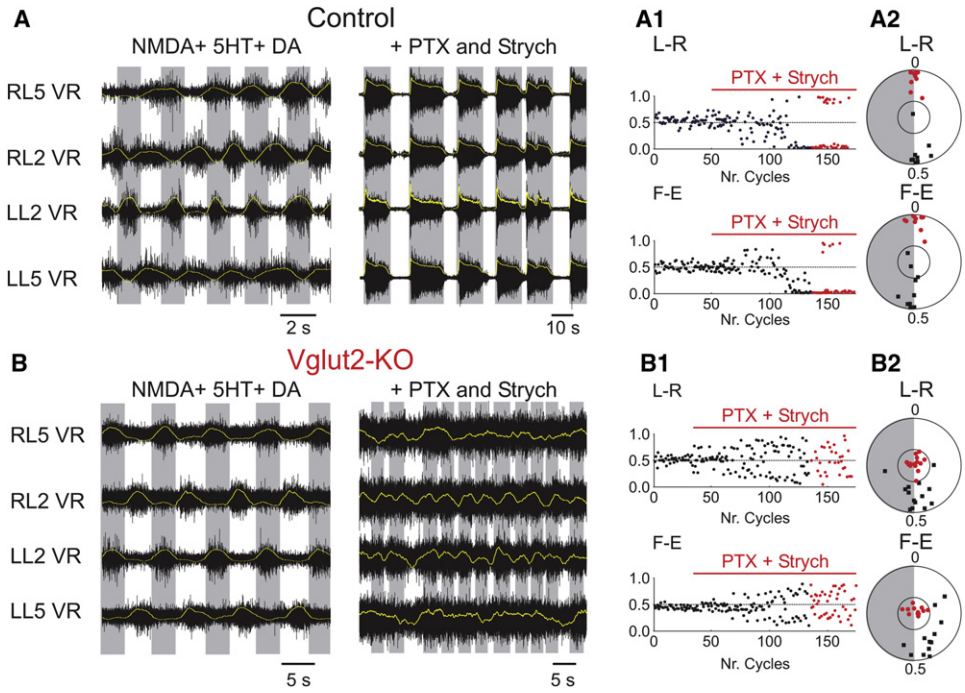


**Figure 6. Effect of Blocking Fast Inhibitory Neurotransmission on Drug-Induced Rhythmic Activity in Vglut2-KO Mice**

(A and B) Locomotor-like activity induced by NMDA-5-HT-DA (10 μM/20 μM/50 μM) in control (A) and Vglut2-KO (B) mice before (left panels) and after blocking $GABA_A$ and glycine receptors with picrotoxin (PTX; 10 μM) and strychnine (0.3 μM), respectively (right panels).

(A1 and B1) Changes in left-right (L-R) and flexor-extensor (F-E) phase coordination plotted for the experiments shown in (A) and (B), respectively.

(A2 and B2) Circular plots of left-right and flexor-extensor coordination in control (A2) and Vglut2-KO (B2) mice before (black squares) and after (red circles) blocking $GABA_A$ and glycine receptors. Note the shift from alternation (0.5) to complete synchrony (0.0) in control mice and the uncoupling in Vglut2-KO mice.

it down and eventually led to the uncoupling of bursts in all roots (Figure 6B: left, control; right, PTX plus strychnine; n = 8, Figure 6B1). This effect is seen as an insignificant coupling between individual roots after blockade of inhibition (Figures 6B1 and 6B2, red circles), and it was observed in all the experiments carried out in Vglut2-KO mice (n = 8). Thus, blockade of inhibition in Vglut2-KO mice resulted in low amplitude and slow frequency oscillations in MNs.

In induced Vglut2-KO mice that otherwise had a locomotor phenotype similar to chronic Vglut2-KO mice, there was a change from alternation into synchronization after total blockade of inhibition, similar to controls (n = 4; data not shown). The 10%–20% remaining Vglut2 protein seen in induced Vglut2-KO was apparently enough to coordinate synchronous activity.

These experiments show that coordination of the drug-induced rhythmic activity observed in chronic Vglut2-KO mice is completely dependent on a $GABA_A$/glycinergic inhibitory network.

## Inhibitory Ia Interneuron and Renshaw Cell Networks Are Present in Vglut2-KO Mice

The experiments blocking inhibitory synaptic transmission suggest that rhythm and pattern generation in the Vglut2 knockout is produced by a network of inhibitory neurons. Well-known inhibitory neurons that provide rhythmic inhibition of MNs during normal locomotor activity are the RCs and the rIa-INs. We first assessed whether the rIa-IN pathway was present in E18.5 Vglut2-KO mice. We took advantage of the recent demonstration that, like cats, newborn mice display a strong Ia-mediated reciprocal inhibition between the knee extensor quadriceps and the knee flexor posterior biceps-semitendinosus (PBST) (Wang et al., 2008). Quadriceps MNs are located in L3, while PBST MNs are located in L5. To demonstrate the reciprocal inhibition, we therefore evoked monosynaptic reflexes in L5 MNs by stimulating the dorsal root (DR) L5 (Figures 7A and 7B, black trace in bottom panel). This stimulation evoked no or little response in the VR L3 (Figure 7B, black trace in top panel). When this stimulation was conditioned by stimulating the DR L3 (2 x T for the monosynaptic reflex recorded in VR L3 (red traces in top panels in Figures 7B and 7C), which should have activated quadriceps-related Ia-INs (Figure 7A), there was a reduction in the amplitude of the L5 monosynaptic reflex (Figure 7B, red trace in bottom panel). We observed inhibition of the DR L5 with conditional stimulus intervals in the range of about 10 ms, similar to what has been reported by Wang et al. (2008) in early newborn animals. The average normalized reduction of the L5-evoked monosynaptic response was 30% ± 6% (n = 6). Vglut2-KO mice showed a similar response (Figure 7C; 31% ± 9%; n = 9).

In the cat spinal cord, activation of RCs by antidromic activation of motor axons causes not only recurrent inhibition of corresponding motor neurons but also inhibition of related Ia interneurons (Hultborn et al., 1971a, 1971b). Thus, activation of extensor RCs, for example, inhibits both extensor MNs and extensor-related Ia-INs exerting inhibition of flexor-related Ia-INs and

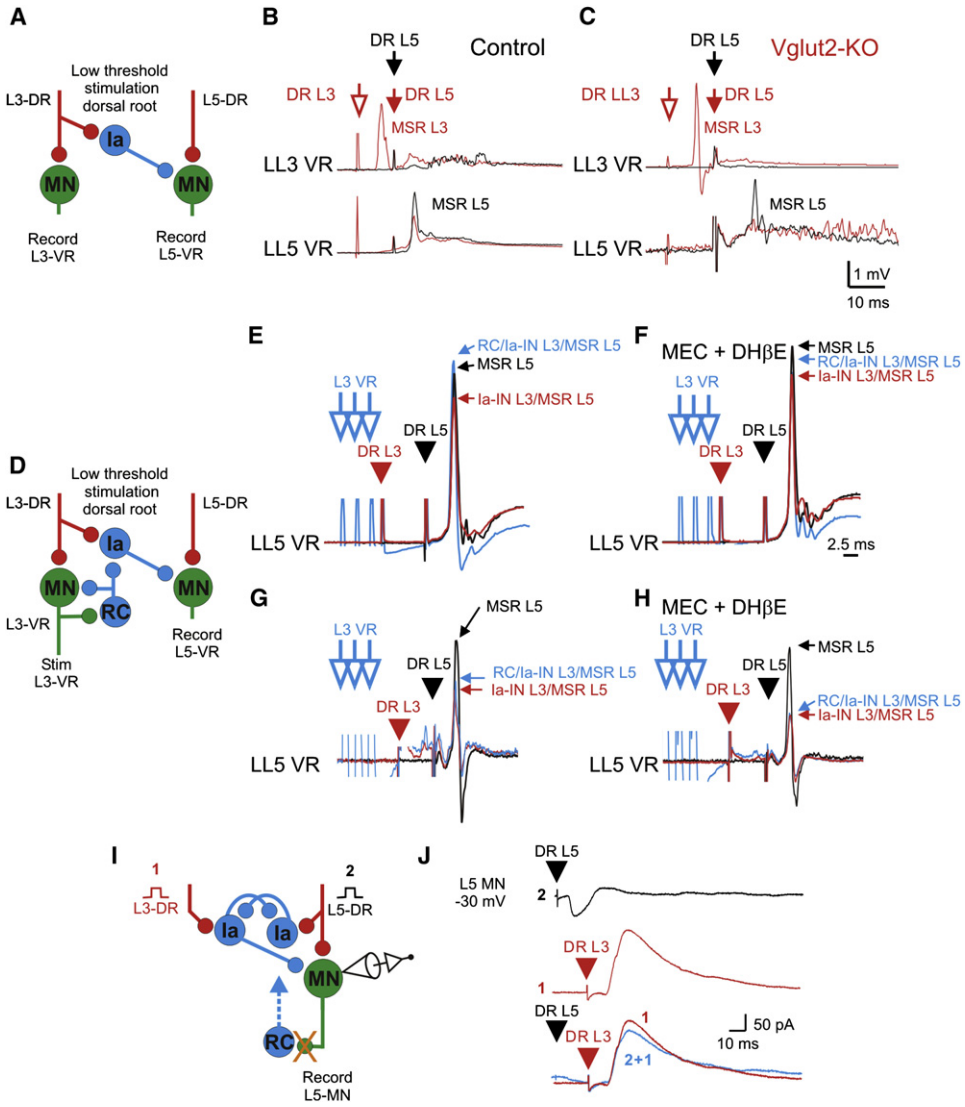


**Figure 7. Reciprocal Ia Interneuron and Renshaw Cell Networks Are Intact in Vglut2-KO Mice**

(A) Schematic of connections between reciprocal Ia-INs and MNs.

(B and C) Recordings from ventral roots L3 (top panels) and L5 (bottom panels) in control (B) and Vglut2-KO (C) mice. The black traces show ventral-root responses following stimulation of the L5 DR at stimulation strengths sufficient to evoke a monosynaptic reflex in L5 VR (MSR L5). Red traces show ventral-root responses when the L5 DR stimulation was conditioned with L3 DR stimulation sufficient to evoke a monosynaptic reflex in the L3 VR (MSR L3). Note the reduction in the amplitude of DR L5 monosynaptic reflex after the conditioning.

(D) Schematic of connections between MNs and RCs and between RCs and rIa-INs.

(E–H) Recording from the ventral root L5 in control (E and F) and Vglut2-KO (G and H) mice with (E and G) or without (F and H) blocking nicotinic receptors with mecamylamine (MEC; 50 μM) and Dihydro-β-erythroidine (DHβE; 50 μM). The black traces show the ventral-root response following stimulation of the L5 DR at stimulation strengths sufficient to evoke a monosynaptic reflex in L5 VR (MSR L5). Red traces show ventral-root responses when the L5 DR stimulation was conditioned with L3 DR root stimulation sufficient to evoke a monosynaptic reflex in the L3 VR (IaIN L3/MSR L5). In the blue traces, the conditioned stimulus was preceded by a train of stimuli to the L3 VR (RC/IaIN L3/MSR L5). Note that the disinhibition of MSR L5 is removed by the nicotinic receptor blockers.

(I) Schematic of setup to demonstrate reciprocal connections between rIa-INs.

(J) Voltage-clamp recording of an L5 motor neuron that receives inhibition from low-threshold L3 DR stimulation (trace 1). Low-threshold L5 DR stimulation (trace 2) attenuates the L3 DR-evoked inhibition (trace 2+1).

flexor MNs (Figure 7D). To test whether RCs can also inhibit Ia-INs in E18.5 mice, we used the same conditional stimulus setup as in Figures 7A–7C but preceded the DR L3 stimulation with a train of L3 VR stimulations. The stimuli applied to VR L3 had durations of 80–150 μs and intensities of 200–500 μA. In this case, the attenuation of the L5 monosynaptic reflex was reduced by 38% ± 16% in control animals (Figure 7E; $p < 0.05$; $n = 3$) and by 46% ± 11% in Vglut2-KO mice (Figure 7G; $p < 0.05$; $n = 5$). This disinhibition was reduced by blocking the transmission from motor neurons to RCs with the nicotinic blockers

mecamylamine (MEC, 50 μM), d-tubocurarine (dTC, 10 μM), or Dihydro-β-erythroidine (DHβE, 50 μM), which reduced it by 95% in control mice (Figure 7F; n = 2) and by 80% in Vglut2-KO mice (Figure 7H; n = 2).

We finally tested whether we could provide evidence for the reciprocal connections between flexor- and extensor-related Ia-INs in the mouse spinal cord. These connections were described directly in the cat spinal cord using recordings from pairs of Ia-INs (Hultborn et al., 1976). Here, we used a more indirect approach and recorded intracellularly from L5 MNs. We reasoned that if we found L5 MNs that received a strong inhibition from low-threshold (1.5−2 × T) stimulation of the DR L3 (flexor-related Ia inhibition of extensor MNs in L5), which could be diminished by low threshold stimulation of the DR L5 (activation of extensor Ia-afferents in L5), it would be an indication of reciprocal inhibition by extensor-related Ia-INs (located in L5) of flexor-related Ia-INs (located in L3). Figure 7J shows such an example with recordings from an L5 MN in an induced Vglut2-KO animal in which the cell was voltage clamped to −30 mV to enhance the amplitude of the DR L3 evoked compound IPSC (trace 1). Stimulation of L5 DR evoked a short latency EPSC (trace 2). When the DR L3 stimulation was preceded by DR L5 stimulation, there was a small but clear reduction of the DR L3-evoked compound IPSC (trace 2+1). Similar findings were seen in two L5 MNs from wild-type mice and two other L5 MNs from induced Vglut2-KO mice with an average reduction of the IPSC by 34% ± 14%.

Together, these experiments show that the reciprocal RC and Ia-IN pathways are present and functional in wild-type and Vglut2-KO E18.5 mice.

### Stimulation of Ventral Roots Blocks Locomotor-Like Activity in Vglut2-KO Mice

Having confirmed that the RC and rIa-IN pathways are present in mice, including the Vglut2-KOs, we directly tested whether components of this network could be the source of the rhythm and flexor-extensor alternation. Given that RCs inhibit Ia-INs, the rhythm should be blocked in Vglut2-KOs by stimulating the ventral root if reciprocal connectivity between flexor- and extensor-related Ia-INs is the source of the rhythm and of the alternating activity between flexor and extensors (Figure 8A; Hultborn et al., 1976; Jordan, 1983).

Prolonged stimulation of the VR (with the same stimulation strength as needed to inhibit the reciprocal Ia pathway; Figure 7) blocked, or strongly attenuated, the ongoing rhythm and the rhythmic oscillations occurring in MNs of Vglut2-KO mice (Figures 8B and 8G; n = 5). This effect was seen when stimulating either L3 or L4 ventral roots (at the same stimulation strength needed to block the reflex) while recording from L2 and L5 (Figure 7G). This effect was blocked by nicotinic receptor blockers (Figure 8C; n = 3). A similar effect was seen in induced Vglut2-KO mice (Figures 8D and 8G; n = 5). In contrast, prolonged stimulation of the ventral roots sped up the rhythm in control mice (Figures 8E and 8F; n = 10), similar to what has been shown before for the disinhibited rhythm in wild-type mice (Bonnot et al., 2009).

Short (1–1.5 s) trains of stimulation of the L4 VRs in Vglut2-KO mice delivered late in the L2 bursting phase caused phase-advance of the next L2 burst with permanent phase shifts of the rhythm (Figure 8H; n = 8 trials in two animals). Similar duration trains delivered in the early phase of the L2 bursts had no effects (n = 12 trials in two animals). Such resetting of the rhythm in a phase-dependent way is indicative of synaptic interaction with the rhythm-generating network (Hultborn et al., 1998).

To test whether RC rhythmic activity was needed for network activity in Vglut2-KO mice, we tested the effect of blocking or reducing the MN-to-RC drive with nicotinic blockers on drug-evoked rhythmic activity. Bath application of mecamylamine did not block the rhythmic activity, although it significantly reduced the frequency of the rhythmic activity elicited by NMDA-5-HT/DA by 21% ± 11% in Vglut2-KO mice (Figure S4; n = 5, $p < 0.001$).

These experiments indicate that the mutual inhibition between opposite rIa-INs is sufficient to coordinate flexor-extensor activity in Vglut2-KO mice.

## DISCUSSION

This study shows that isolated spinal cords from Vglut2-KO mice, in the apparent absence of neuronal glutamate release in the locomotor region of the spinal cord, are able to produce rhythmic motor outputs in the presence of neurotransmitter agonists. This rhythmic activity shares similarities with the locomotor-like activity observed in control mice with flexor-extensor and left-right alternation. However, unlike wild-type mice, concomitant blockade of $GABA_A$ and glycinergic inhibition completely uncouples the coordination in Vglut2-KO mice. Like in adult cats and newborn wild-type mice, Renshaw cell and Ia interneuron networks are present in Vglut2-KO mice. In the absence of Vglut2-mediated excitatory neurotransmission, reciprocally coupled Ia interneuron networks appear sufficient to generate the rhythm and to coordinate the flexor-extensor alternation.

### Inactivating the Gene Encoding for Vglut2 Prevents Intrinsic Glutamate Release in the Spinal Cord

The procedure for making the Vglut2 gene nonfunctional is similar to that used previously in that the Vglut2 gene was rendered inactive using conditional excision of one or several exons in the gene (Moechars et al., 2006; Wallén-Mackenzie et al., 2006). These mice, including the Vglut-KO mice used in the present study, showed elimination of the Vglut2 protein in the central nervous system, causing a perinatal lethal phenotype due to lack of respiratory drive. Here, we additionally show that the lack of Vglut2 protein resulted in a severe reduction in spontaneous release of glutamate onto MNs and neurons in the ventral spinal cord and a complete abolishment of stimulus-evoked Vglut2-mediated glutamate release in the spinal cord. The loss of Vglut2 protein was not compensated for by quantitative changes in the protein levels of the known vesicular glutamate/aspartate (Vglut1, Vglut3, Sialin), cholinergic (VaChT), or GABA/glycine transporters. These findings suggest that glutamate transmission is absent or very severely reduced in the population of Vglut2-positive spinal neurons. Because all glutamatergic neurons in the ventral spinal cord exclusively express Vglut2, the Vglut2-KO mouse is an experimental model in which

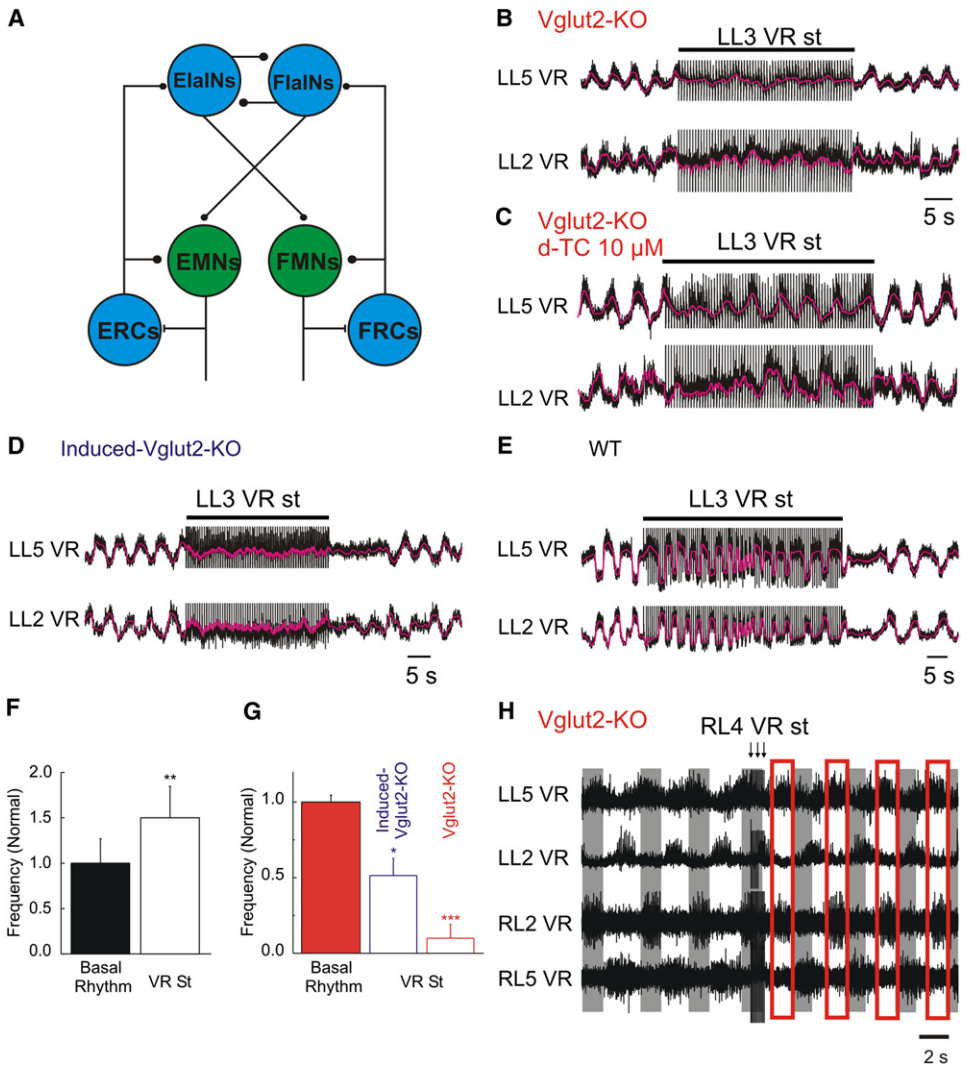


**Figure 8. Activation of Ventral Roots Suppresses Drug-Induced Locomotor-Like Activity in Vglut2-KO Mice**

(A) Schematic of flexor- and extensor-related RCs and rIa-IN connections.

(B–E) Ventral-root recordings showing drug-induced rhythmicity in Vglut2-KO (B–C), induced Vglut2-KO (D), and control (E) mice. Prolonged trains of stimuli applied to the ventral root totally abolish the rhythm in the Vglut2-KO (B) and the induced Vglut2-KO (D) mice. This effect is suppressed by blockade of nicotinic receptors with D-tubocurarine (d-TC, 10 μM) (C). Ventral-root stimulation accelerated the rhythm in control mice (E).

(F and G) Normalized changes in frequency induced by ventral-root stimulation in wild-type (F) and induced Vglut2-KO/Vglut2-KO (G) mice (*$p < 0.02$, **$p < 0.01$, ***$p < 0.001$). Error bars represent ± SEM.

(H) A short train of stimulation to the ventral root may cause permanent phase shifts of the ongoing rhythm in Vglut2-KO mice. The gray shaded boxes indicate the VR RL2 activity before stimulation and the expected timing after stimulation, whereas the red boxes indicate the actual VR RL2 activity after stimulation. VR LL2 and LL5 were recorded in DC mode.

See also Figure S4.

there is no glutamate release within the intrinsic spinal motor networks and in which the only functional glutamate release is from proprioceptive primary afferents that employ Vglut1 (Hughes et al., 2004; Pecho-Vrieseling et al., 2009).

## Purely Inhibitory Networks Can Generate Locomotor-Like Activity in Vglut2-KO Mice

It was impossible to evoke locomotor-like activity in the Vglut2-KO mice either by descending or afferent fiber stimulation. These findings identify Vglut2-mediated neurotransmission as being indispensable for neural-evoked locomotor activity because Vglut2-positive neurons are essential elements of the supraspinal and afferent activation systems and/or because Vglut2-expressing neurons in the spinal cord are the targets of these neural systems. The inability to evoke locomotion by activating glutamatergic neurons directly in the spinal cord of Vglut2-KO shows that when glutamate release is intrinsically blocked in Vglut2-expressing neurons, these cells no longer contribute to generation of locomotor activity. Despite the absence of neural-evoked locomotor-like activity, we succeeded in evoking

rhythmic activity with external application of neuroactive substances in the Vglut2-KO mice, similarly to what was briefly described previously in another line of Vglut2-KO mice (Wallén-Mackenzie et al., 2006). Our experiments unambiguously show that Vglut2-KO mice can display drug-induced rhythmic activity that has similarity to normal locomotor-like activity observed in isolated spinal cords from wild-type mice but that has a higher threshold for initiation and a lower frequency range.

Because chronic transmitter ablation from the spinal cord may lead to developmental changes in the assembly of spinal circuits, we also eliminated the Vglut2 protein close to the day of experiments. As reported previously in studies using inducible Cre recombination, we found an elimination of 80%–90% of the protein product of the target gene (Chow et al., 2006). Despite the fact that the Vglut2 protein was not completely eliminated in the spinal cord, these animals showed a locomotor phenotype similar to the chronic Vglut2-KO mice, suggesting that the network is representing an assembly of neurons that is configured in a way similar to those seen in wild-type.

The drug-induced locomotor-like activity in chronic Vglut2-KO mice was interrupted by a blockade of fast GABAergic and glycinergic neurotransmission that excluded a functional role for excitatory neural networks as a source of rhythm generation in the Vglut2-KO mice. Rather, the locomotor network has been reduced to an inhibitory network that can produce an alternating rhythmic motor activity when appropriately driven by neuroactive substances, independent of intrinsic neuron-to-neuron glutamate receptor activation.

### Networks Generating Locomotor-Like Activity without Active Vglut2-Positive Neurons

Miller and Scott (1977) proposed a rhythm- and pattern-generating model for mammalian locomotion based on the known connectivity between groups of the inhibitory RCs and rIa-INs (Figure 8A; Hultborn et al., 1971a, 1971b, 1976). In the model, tonic excitation of rIa-INs converts the two groups of Ia-INs into a bistable circuit in which one group is active and the other inactive. The Miller and Scott model is considered to be insufficient to explain rhythm and pattern generation underlying normal mammalian locomotion. Thus, ventral-root stimulation (that antidromically activates RCs and inhibits rIa-INs) does not block or attenuate the frequency of the rhythm in the cat (Jordan, 1983) or in wild-type rodents (Bonnot et al., 2009). On the contrary, ventral-root stimulation speeds up both the disinhibited rhythm (Bonnot et al., 2009) and the drug-induced locomotor-like activity (Figure 8), suggesting that glutamate release from MN terminals (Mentis et al., 2005; Nishimaru et al., 2005) enhances locomotor activity (by a still-unknown mechanism). In contrast, in the Vglut2-KO mice, ventral-root stimulation completely blocks or severely reduces the frequency of the rhythmic activity. Moreover, the locomotor rhythm persisted when MN inputs to RC were reduced by nicotinic blockers. Together, these experiments strongly suggest that the rIa-INs act as mutually inhibitory cores for generating the rhythm in the Vglut2-KO mice. Interestingly, the connectivity patterns between RCs and rIa-INs predict that blocking activation of RCs by nicotinic antagonists should slow down the rhythm but not block the rhythmic activity or flexor-extensor alternation. This is indeed what we observed in the present experiments. The ability to reset the ongoing rhythm in Vglut2-KO mice with short trains of stimuli to the ventral root (Figure 8H) is a further indication that in these mice the RC cells directly access the rhythm-generating core.

Thus, our experiments provide strong evidence that a reciprocally connected Ia-IN network (that is directly connected to MNs) may generate a rhythm and also that their activity is sufficient to explain the flexor-extensor coordination in the Vglut2-KO mice when stimulated by drugs. A role for rIa-IN contribution to flexor-extensor alternation during locomotion has long been proposed (see references in Geertsen et al., 2011). However, attempts to link rIa-INs to flexor-extensor alternation using genetic ablation of molecularly defined inhibitory neurons that encompass rIa-INs have failed thus far (Gosgnach et al., 2006), although ablation of most of the ipsilaterally projecting inhibitory interneurons in the spinal cord (Zhang et al., 2010, Soc. Neurosci., abstract) upset flexor-extensor alternation. By taking advantage of the known connectivity pattern between RCs and rIa-INs and eliminating the excitatory neurons from the network, we demonstrate that the rIa-IN network may be sufficient to generate flexor and extensor alternation.

In the present study, we did not record directly from RCs and rIa-INs during drug-induced locomotion. In cat (Noga et al., 1987) and newborn mice (Nishimaru et al., 2006), the rhythmic modulation of RCs is severely reduced in the presence of nicotinic receptor blockers. We therefore expect that in the Vglut2-KO mice RCs are also mainly driven by MNs. Moreover, we show that RCs are not essential for rhythm generation and flexor-extensor alternation because a blockade of the cholinergic receptors in Vglut2-KO mice does not suppress the rhythm (Figure S4). Recordings from rIa-INs would be of interest in order to determine whether flexor-related and extensor-related Ia-INs fire in the appropriate phase to generate the observed flexor-extensor alternation in the Vglut2-KO mice. However, simultaneous intracellular recordings from identified pairs of rIa-INs and MNs that are necessary to directly reveal the reciprocal firing pattern are extremely demanding and were therefore not pursued in the present study.

Another issue regarding the inhibitory network is that GABA and glycine may have depolarizing rather than hyperpolarizing actions at E18.5 because of the chloride reversal potential. Indeed, it was shown that the chloride reversal potential is above the resting membrane potential at E18.8 in mice (Delpy et al., 2008). However, even though GABA/glycine may depolarize MNs until E.18.5, this depolarization fails to trigger action potentials and may act as shunting inhibition (Jean-Xavier et al., 2007; O'Donovan, 1989). Here we do see functional inhibition of dorsal root-evoked MN responses (Figure 7). Moreover, during drug-induced locomotor-like activity in which the motor neuron and interneuron membrane potentials are depolarized by the combined action of the drugs, we see functional inhibition of network activity from stimulating the ventral root (Figure 8).

While flexor-extensor alternation can be readily explained by the physiologically established connections between rIa-INs and inhibitory connections to MNs, it is not possible to explain the preserved left-right coordination with these connections. Obviously, crossed connections are needed for this to happen (Kiehn, 2011). One possibility is that some lumbar rIa-INs are

also commissural, as described for sacral Ia-INs in the cat spinal cord (Jankowska et al., 1978). In this case, rIa-INs may be reciprocally connected not only ipsilaterally, but also commissurally, and they may regulate both flexor-extensor and left-right alternation in the Vglut2-KO mice. An alternative possibility is that inhibitory commissural interneurons (CINs) connect to rIa-INs on the other side of side of the cord. Such connections exist for excitatory CINs (Jankowska, 2008; Quinlan and Kiehn, 2007) but have so far not been described for inhibitory CINs, although such projections to RCs have been revealed (Nishimaru et al., 2006).

### Are Reciprocal Ia Inhibitory Interneuron Networks the Only Source of Flexor and Extensor Motor Coordination?

There are inhibitory neurons in the spinal cord other than rIa-INs that project directly to MNs, including nonreciprocal Ia-INs and commissural inhibitory neurons. These groups of neurons have been shown to be rhythmically active during drug-induced locomotor-like activity (Quinlan and Kiehn, 2007; Wilson et al., 2010). We therefore do not exclude a role for them in providing rhythmic synaptic inputs to MNs during locomotion, although they are not affected by RC inhibition or organized in reciprocal connectivity patterns (Hultborn et al., 1971a, 1971b). Inhibitory neurons may also play a role in providing reciprocal activity between rhythm-generating excitatory neurons that are upstream from the inhibitory network in a locomotor network with intact glutamatergic transmission (see below).

### Rhythm Generation in the Vglut2-KO Mice Depends on Chemical Activation of the Inhibitory Network

In contrast to control mice, locomotor-like activity could only be elicited in Vglut2-KO mice when NMDA was applied together with 5-HT and DA. NMDA receptor activation could not be substituted with non-NMDA glutamate receptor activation. This dependence on NMDA receptor activation for induction of locomotor-like activity suggests that the burst-like properties that NMDA receptor activation can evoke in spinal neurons (Hochman et al., 1994; Ziskind-Conhaim et al., 2008) is a requirement for rhythm generation in the Vglut2-KO mice. The rhythm generation, therefore, seems to be a consequence of the interplay between cellular rhythmogenic properties and reciprocal inhibitory coupling between groups of inhibitory neurons, similar to what has been observed in many invertebrate motor networks (Marder and Calabrese, 1996) and in the mammalian cortex (Bartos et al., 2007). The fact that slow low-amplitude oscillations were seen in individual root recordings after blocking inhibition suggests that even MNs may display NMDA-induced oscillations, similarly to what was previously described (Tresch and Kiehn, 2000).

Notably, the frequency of the rhythm in Vglut2-KO mice was restricted to the lower part of the frequency spectrum (<0.4 Hz) reported for locomotor-like activity in the neonatal mouse (Talpalar and Kiehn, 2010), and for any given drug concentrations the frequency always remained lower in the Vglut2-KO compared to the control littermates. These observations suggest that, although the rIa-IN networks can generate a rhythm, this is not the normal state in an intact locomotor network. Rather, the rIa-INs may be driven into rhythmicity by upstream flexor- and extensor-related excitatory CPG neurons (Kiehn, 2011; McCrea and Rybak, 2008). These rhythmogenic excitatory networks may be connected via inhibitory neurons that are different from the rIa-INs (see Kiehn, 2011). We anticipate that under normal circumstances these excitatory circuits produce and pace the rhythm. However, when the Vglut2-dependent neurotransmission is removed, the rhythm generation can be shifted to the Ia inhibitory networks. These latter networks could only be brought to bursting when stimulated with drugs and could not be accessed by the neural locomotor initiating signals. In this sense, the rhythmogenic capability of Ia inhibitory networks in the Vglut2-KO mice is a consequence of the removal of the excitatory network components. Our experiments, therefore, stress the need for a careful and intervening analysis in order to understand the significance of changes in network structure when mouse mutants are investigated in the in vitro conditions and when locomotor-like activity is induced by drugs.

## EXPERIMENTAL PROCEDURES

### Mice

The details of generating the Vglut2 knockout mice are reported elsewhere (Supplemental Experimental Procedures; Hnasko et al., 2010). The generation and specificity of the BAC-Vglut2-Chr2-YFP mouse is described in Hägglund et al. (2010). ROSA26-Cre-ER™ mice were obtained from Jackson Laboratory. The procedure for inducing Cre is described in the Supplemental Experimental Procedures.

### Western Blot Analysis

Protein levels for Vglut1, Vglut2, Vglut3, Sialin, VAChT, VIAAT, and actin were measured in spinal cords and brains from either wild-type or mutant E18.5 mice using western blot analysis (Supplemental Experimental Procedures).

### Preparation for Electrophysiological Experiments

All experimental procedures were approved by the local animal care and ethical committee. Spinal cords from E18.5 mice were isolated (Supplemental Experimental Procedures). The embryonic stage was designated E0.5 on the morning of plug formation. The neural axis was cut either at C1 and at S1 or rostrally between the mesencephalon and the diencephalon and caudally at S4. The isolated nervous system was transferred to a recording chamber continuously perfused with normal Ringer's solution containing 111 mM NaCl, 3 mM KCl, 11 mM glucose, 25 mM $NaHCO_3$, 1.25 mM $MgSO_4$, 1.1 mM $KH_2PO_4$, and 2.5 mM $CaCl_2$ and saturated with 95% $O_2$/5% $CO_2$ for a pH of 7.4. All recordings were done at room temperature (22°C–24°C).

### Recordings from Motor Neurons and RCs

Whole-cell recordings were obtained from visually patched MNs and interneurons medial to the MNs located in the same segments as the recorded ventral roots (Nishimaru et al., 2006; Nishimaru et al., 2005). MNs were identified by antidromic activation from the ventral roots before QX-314 diffused enough to block action potentials. RCs were identified by generation of short-latency nicotinic EPSPs upon stimulation of the nearest ventral root (Supplemental Experimental Procedures).

### Recordings from Nonmotor Neurons in the Ventral Spinal Cord

Unidentified neurons recorded outside the motor nucleus were blindly patched for intracellular recordings (Supplemental Experimental Procedures).

### Recording of Motor Activity

Motor activity was recorded in ventral roots with suction electrodes attached to the lumbar ventral roots (VRs) L2 and L5 on the left and the right side of the cord (Supplemental Experimental Procedures).

### Electrical Stimulation of Descending and Afferent Fibers

The protocol for stimulating descending and afferent fibers for inducing locomotor-like activity was similar to the one employed in previous studies (Supplemental Experimental Procedures; Zaporozhets et al., 2004).

### Drugs

The following glutamate agonists were used in combination with serotonin (5-HT) and dopamine (DA): N-methyl-D-aspartate (NMDA), kainate, and (RS)-2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4yl) propanoic acid (ATPA;Tocris). The following glutamate receptor antagonists were used: 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) and D-(-)-2-amino-5-phosphonopentanoic acid (AP5). Nicotinic receptors were blocked with mecamylamine, Dihydro-β-erythroidine hydrobromide (Tocris), and d-Tubocurarine. $GABA_A$ and glycine receptors were blocked with picrotoxin and strychnine, respectively. All drugs were purchased from Sigma if not otherwise specified.

### Dorsal- and Ventral-Root Stimulation

Monosynaptic reflexes were evoked by stimulating dorsal roots, and the stimulus strength was graded as multiples of the threshold (T) responses recorded in the ventral roots.

### Data Analysis of Rhythmic Activity

Data points for analyzing cycle periods and burst amplitudes were taken after the locomotor activity had stabilized 10–15 min after the initial burst of activity. Flexor-extensor and left-right coordination were evaluated with circular statistic, in which the vector direction gives the preferred phase of the activity and the length of the vector (r) the precision of the phase. p values larger than 0.05 determined by Rayleigh's test were considered nonsignificant (Supplemental Experimental Procedures).

### Statistics

For comparison between means, we used either two-sample unequal variance Student's t test (unpaired data) or paired Student's t test (paired data). Data are reported as means and SEM, unless otherwise noted.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.07.011.

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