Spinal Hb9::Cre-derived excitatory interneurons contribute to rhythm generation in the mouse

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Rhythm generating neurons are thought to be ipsilaterally-projecting excitatory neurons in the thoracolumbar mammalian spinal cord. Recently, a subset of Shox2 interneurons (Shox2 non-V2a INs) was found to fulfill these criteria and make up a fraction of the rhythm-generating population. Here we use Hb9::Cre mice to genetically manipulate Hb9::Cre-derived excitatory interneurons (INs) in order to determine the role of these INs in rhythm generation. We demonstrate that this line captures a consistent population of spinal INs which is mixed with respect to neurotransmitter phenotype and progenitor domain, but does not overlap with the Shox2 non-V2a population. We also show that Hb9::Cre-derived INs include the comparatively small medial population of INs which continues to express Hb9 postnatally. When excitatory neurotransmission is selectively blocked by deleting Vglut2 from Hb9::Cre-derived INs, there is no difference in left-right and/or flexor-extensor phasing between these cords and controls, suggesting that excitatory Hb9::Cre-derived INs do not affect pattern generation. In contrast, the frequencies of locomotor activity are significantly lower in cords from Hb9::Cre-Vglut2Δ/Δ mice than in cords from controls. Collectively, our findings indicate that excitatory Hb9::Cre-derived INs constitute a distinct population of neurons that participates in the rhythm generating kernel for spinal locomotion.

Locomotor behaviors, such as flying, swimming or walking, are complex motor actions that give animals and humans the ability to move and interact with the surroundings. The generation of locomotion in vertebrates is largely determined by neural networks in the spinal cord1–4. These spinal networks are responsible for key features that characterize limb locomotion in mammals, rhythm generation and the precise coordination of muscle activation. In the ventral spinal cord where the locomotor circuit is located5, developmentally expressed transcription factors6–7 have allowed for the identification and targeted manipulation of selected interneuron populations3,8–10. These studies have provided insights into the organization of the neuronal circuits underlying left-right11–16 and flexor-extensor17–19 coordination in mammals. Optogenetic, pharmacological, and lesion studies have provided strong evidence that the rhythmic drive in the mammalian spinal locomotor circuit comes from activity in ipsilaterally-projecting excitatory glutamatergic neurons1–3,9,20–24. However, despite the ablation of entire progenitor domain-derived classes of ipsilaterally-projecting excitatory neurons, rhythm-generating interneurons still remain elusive12,14,16,25–27. Therefore, there has also been focus on identifying and studying cell populations that span several progenitor domains. Recently, one such study directly linked rhythm generation to a group of excitatory interneurons identified by the embryonic expression of the transcription factor short stature homeobox protein 2 (Shox2)28. Shox2 neurons span several of the dorsally and ventrally progenitor domain-derived interneuron classes, and partially overlap with V2a neurons which express the transcription factor Chx10 during development28–30. Shox2 non-V2a neurons were shown to be involved in rhythm generation28. However, eliminating Shox2 non-V2a neurons from the network did not result in complete abolition of locomotor rhythm. Collectively, these data suggest that glutamatergic interneuron populations other than those captured in the current transcription factor schemas are involved in rhythm generation. To target such populations we are examining currently available genetic tools that may define unique neuronal groups to which we can ascribe such a function.

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In the current study, we selectively silence glutamatergic synaptic transmission in Hb9::Cre-derived excitatory neurons while retaining motor neuron output. We demonstrate that although reporter expression observed in Hb9::Cre mice represents a much larger population of neurons than those expressing the Hb9 protein postnatally, the population identified is consistent and can be reliably used to target and manipulate a novel excitatory neuronal population in the spinal cord. We also show that excitatory Hb9::Cre-derived interneurons do not overlap with the Shox2 non-V2a population. Synaptically silencing the excitatory subset of Hb9::Cre-derived interneurons by a targeted deletion of the vesicular glutamate transporter 2 (Vglut2) leads to a significant reduction in locomotor frequency without any significant effect in pattern formation, suggesting a role in rhythm generation.

Results

Hb9::Cre-derived INs are located in the ventral and dorsal spinal cord. Although Hb9::Cre and Hb9-GFP mice were generated with an analogous strategy to the Hb9-LacZ mice, reporter expression in Hb9::Cre-reporter-labeled (Hb9::Cre::Rosa26-FP) and Hb9-GFP mice is not restricted to motor neurons (MNs). In the Hb9-GFP mouse, a ventral population of interneurons is marked, whereas in the Hb9::Cre::Rosa26-FP mouse line a dorsal population of cells is also captured. The increase in the number and laminar distribution of fluorescent reporter cells observed in Hb9::Cre::Rosa26-FP mice may be attributed to the transient embryonic expression of Hb9 in these cells. 

YFP expression in Hb9::Cre::Rosa26-YFP mice was detected through lamina I to VI in addition to lamina VII, VIII and ventral X (Fig. 1A) throughout the lumbar spinal cord. This is in contrast to the GFP expression in Hb9-GFP mice, which is restricted to lamina VII, VIII and ventral X (Fig. 1B). We will collectively refer to these dorsal (lamina I-VI) and ventral neuronal populations in Hb9::Cre::Rosa26-FP mice as Hb9::Cre-derived INs.

Despite the larger number of reporter-expressing cells in Hb9::Cre::Rosa26-FP mice, reporter expression identifies a consistent group of interneurons throughout the lumbar spinal cord. Thus, we use the Hb9::Cre mouse as a genetic tool to study the possible roles of excitatory Hb9::Cre-derived INs in locomotion.

One group of interest is the group of neurons labeled by the transcription factor Hb9. Hb9 is usually described as a postmitotic motor neuron marker, however, it is also transiently expressed in a larger group of progenitor cells and postmitotic neurons embryonically. As it is commonly done when targeting spinal cord interneuron populations, we use conditional genetics and rely on Cre-dependent recombination to reliably target excitatory interneurons identified by the developmental expression of Hb9. This approach includes the small medial subset of interneurons retaining Hb9 protein expression postnatally, which have been extensively studied and found to be excitatory neurons displaying cellular properties consistent with being potential rhythm generating neurons.
Canonical Hb9 INs account for less than 1% of the Hb9::Cre-derived IN population. We first asked if the Hb9::Cre-derived INs include the canonical Hb9 INs. We define canonical Hb9 INs as the small sub-set of neurons clustered in medial lamina VIII in the lower thoracic and upper lumbar mouse spinal cord. These interneurons retain endogenous HB9 protein expression postnatally and also co-express GFP protein under the Hb9 promoter in Hb9-GFP mice (type I cells referred in refs 33 and 35) (Fig. 1B, white boxed area). These canonical Hb9 neurons have been suggested to be part of the kernel for rhythm generation in the mammalian locomotor network33–36.

In Hb9::Cre:Rosa26-YFP mice, an overlap of YFP and HB9 protein was evident in canonical Hb9 INs, motor neurons (MNs) and sympathetic preganglionic neurons (Fig. 1A). We indeed found that the majority of canonical Hb9 INs co-express HB9 protein and the reporter protein, YFP, in Hb9::Cre:Rosa26-YFP mice (86% ± 7%, N = 3, 18 sections). Conversely, canonical Hb9 INs make up less than 1% (0.86% ± 0.37%) of the Hb9::Cre-derived IN population (N = 3, 18 sections) (Fig. 1C). The number of canonical Hb9 neurons (193 ± 61 cells between Th12 and L3, N = 3) is similar to what was previously reported33. Taken together these data indicate that most of the canonical Hb9 INs are part of Hb9::Cre-derived IN population, but they account for a very small percentage of this population. Therefore, hereinafter whenever we refer to the Hb9::Cre-derived INs, the canonical Hb9 INs in Th12-L3 will be implicitly included.

One third of Hb9::Cre-derived INs are glutamatergic. We next sought to assess the transmitter phenotype of the Hb9::Cre-derived IN population by examining overlap of reporter expression in Hb9::Cre:Rosa26-YFP, Vglut2-GFP, Hb9::Cre:Rosa26-YFP, Glyt2-GFP, and Hb9::Cre:Rosa26-YFP;Gad67-GFP mice. We defined glutamatergic neurons as those co-expressing Vglut2-GFP and tdTomato, and glycinergic neurons as those co-expressing Glyt2-GFP and tdTomato. We found that one third of the Hb9::Cre-derived INs are glutamatergic (Vglut2-GFP+, 33% ± 2%), GABAergic (Gad67-GFP+, 34% ± 1%), and glycinergic (Glyt2-GFP+, 33% ± 1%) in Hb9::Cre:Rosa26-YFP spinal cords.
mice, in order to identify Hb9::Cre-derived glutamatergic (Fig. 2A), glycinergic (Fig. 2B), and GABAergic (Fig. 2C) neurons, respectively. Motor neurons are also labeled by Isl1 antibody (blue box). Rightmost pictures are magnifications of the white boxed area. Arrowheads indicate overlap between Isl1 (red) and Hb9::Cre-derived INs (green). Scale bars: 100 μm and 50 μm.

Figure 3. Hb9::Cre-derived INs do not overlap with the Shox2 non-V2a population. (A) Co-expression of YFP (green) and Isl1 antibody (red) in the Hb9::Cre;Rosa26-YFP mouse spinal cord at E11.5. Motor neurons are also labeled by Isl1 antibody (blue box). Rightmost pictures are magnifications of the white boxed area. Arrowheads indicate overlap between Isl1 (red) and Hb9::Cre-derived INs (green). Scale bars: 100 μm and 50 μm. (B) Co-expression of YFP (green), Shox2 antibody (red) and/or Chx10 antibody (blue) in the Hb9::Cre;Rosa26-YFP mouse ventral spinal cord at E11.5. Rightmost pictures are magnifications of the white boxed area. Arrowheads indicate overlap between Hb9::Cre-derived INs (green) and Shox2+ Chx10− (red), Shox2− Chx10+ (blue) or Shox2+ Chx10+ (pink). Scale bars: 100 μm and 50 μm. (C) Quantification of overlap in (A) and (B). Bar graph showing percent of overlap between Hb9::Cre-derived INs (green) and Shox2 non-V2a (Shox2+ Chx10−, 1.3% ± 0.2%), and Isl1 (Isl1+, 6% ± 0.2%) INs. Error bars represent ± SEM. (D) Percent of the Shox2 non-V2a IN population (Shox2+ Chx10−) that overlaps with Hb9::Cre-derived INs (YFP+) in the Hb9::Cre;Rosa26-YFP mouse spinal cord at E11.5. Shox2 non-V2a INs rarely co-express YFP (Shox2+ YFP+, darker grey) (12% ± 2%). Error bars represent ± SEM.

Hb9::Cre-derived INs span several progenitor domains, but constitute a population distinct from the Shox2-nonV2a INs. To further characterize the excitatory Hb9::Cre-derived IN population, we turned to the developmentally expressed transcription factors found in excitatory neurons, and looked for overlap...
with the exclusively excitatory and ipsilateral IN markers, Isl1, Chx10 and Shox2 (Fig. 3A and B respectively). All experiments were performed at embryonic stage E11.5 to capture the peak of expression of these early neuronal markers.

The LIM homeodomain transcription factor 1 (Isl1) is expressed in MNs and is involved in MN differentiation\(^1\). Its expression defines a class of interneurons, the dI3 INs\(^{43,44}\), which have been shown to be involved in the cutaneous regulation of paw grasp\(^2\) but not in locomotor rhythm or patterning. Therefore, we sought to investigate whether or not dI3 INs and Hb9::Cre-derived INs were overlapping. We found that Hb9::Cre-derived INs seldomly co-expressed Isl1 (6% ± 0.2%, N = 2, 22 sections) (Fig. 3C), indicating that the dI3 IN population shows no appreciable overlap with the Hb9::Cre-derived INs.

Chx10 is the marker for V2a neurons and the expression of Shox2 and Chx10 can be used to categorize excitatory neurons in the ventral spinal cord into three groups: Shox2 V2a INs (Shox2\(^+\) Chx10\(^−\)), Shox2 non-V2a INs (Shox2\(^+\) Chx10\(^−\)), and Shox2 OFF V2a INs (Shox2 \(^+\) Chx10\(^+\))\(^{28}\). Shox2 non-V2a INs is the only subgroup of excitatory neurons that has been shown to contribute to rhythm generation in mammals, whereas Shox2 V2a INs, and Shox2 OFF V2a INs seem to be involved in pattern generation\(^{28}\). We found that Hb9::Cre-derived INs rarely overlapped with the Shox2 V2a (4% ± 0.1%), Shox2 OFF V2a (2% ± 0.1%) and Shox2 non-V2a (1.3% ± 0.2%) populations (N = 2, 22 sections) (Fig. 3C). Moreover, we also verified that Hb9::Cre-derived INs make up less than 12% ± 2% of the Shox2 non-V2a population (Fig. 3D).

Altogether, these data indicate that although excitatory Hb9::Cre-derived INs are a heterogeneous group of neurons that span several excitatory progenitor domains, they make up a distinct group of excitatory neurons that only marginally overlaps with the Shox2 non-V2a population.

**Vglut2 is effectively removed from excitatory Hb9::Cre-derived INs in Hb9::Cre-Vglut2\(^{Δ/Δ}\) mice.**

In order to selectively target excitatory Hb9::Cre-derived INs and investigate their role in locomotor network activity, Hb9::Cre mice were crossed with mice carrying a conditional floxed Vglut2 allele to produce offspring with a Cre-dependent selective loss of Vglut2 (see Experimental Procedures), thereby disrupting synaptic transmission exclusively in excitatory Hb9::Cre-derived INs. As shown in previous work from our lab and others, removal of Vglut2 efficiently blocks action potential mediated synaptic transmission from the affected neurons\(^{17,26,28}\), and hence results in the functional removal of these neurons from the network.

To assess the loss of Vglut2 transcript, we performed in situ hybridization with a Vglut2 probe on spinal cord tissue from both mice which have complete removal of Vglut2 from Hb9::Cre-derived neurons (Hb9::Cre::Vglut2\(^{Δ/Δ}\); Rosa26-YFP, referred to as Hb9::Cre-Vglut2\(^{Δ/Δ}\)) and littermates which have one copy of Vglut2 removed but retain normal synaptic function (Hb9::Cre::Vglut2\(^{Δ/Δ}\); Rosa26-YFP, referred to as controls). Vglut2 signal was seen extensively throughout the gray matter in cords from both control (Fig. 4A) and Hb9::Cre-Vglut2\(^{Δ/Δ}\) mice (Fig. 4B). However, Vglut2 mRNA expression was reduced by 81% (N = 3 animals per condition, 26 sections), in Hb9::Cre-derived INs, in Hb9::Cre-Vglut2\(^{Δ/Δ}\) ventral spinal cords compared to controls.

**Silencing the synaptic signaling of excitatory Hb9::Cre-derived INs reduces the frequency of locomotor-like activity.** To determine the functional impact of the loss of excitatory synaptic transmission

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**Figure 4. Loss of Vglut2 expression in Hb9::Cre-Vglut2\(^{Δ/Δ}\) mice.** (A and B) Fluorescent in situ hybridization shows co-localization of Vglut2 mRNA (red) and Hb9::Cre-derived INs (YFP, green) in control (Hb9::Cre::Vglut2\(^{Δ/Δ}\); Rosa26-YFP) (A), and Hb9::Cre-Vglut2\(^{Δ/Δ}\) (Hb9::Cre::Vglut2\(^{Δ/Δ}\); Rosa26-YFP) (B) P0 spinal cords. Rightmost pictures are magnifications of the white boxed areas. Asterisks indicate Vglut2 mRNA in Hb9::Cre-derived INs. Scale bars represent 100 μm for the transverse hemi-sections and 50 μm for the magnified boxes on the right.
in Hb9::Cre-derived INs, we performed locomotor experiments in spinal cords isolated from early postnatal animals. Although Hb9::Cre-Vglut2Δ/Δ mice appear to breathe and have spontaneous movements, they do not survive to P1. Therefore, all experiments were performed at P0. Locomotor-like activity was evoked by bath application of NMDA and serotonin (5-HT). The concentration of 5-HT was kept at 8 μM, while the NMDA was varied (5 μM, 7 μM, and 10 μM) in order to test a range of locomotor frequencies. Locomotor-like activity was evoked in all controls (N = 13/13) (Fig. 5A, upper traces) and Hb9::Cre-Vglut2Δ/Δ cords (N = 11/16; 7 μM NMDA, N = 16/16) (Fig. 5A, lower traces). However, locomotor frequencies in Hb9::Cre-Vglut2Δ/Δ cords were 26–31% lower than that of controls at all NMDA concentrations tested (p < 0.0001) (controls: 0.27 ± 0.02 Hz at 5 μM, 0.33 ± 0.01 Hz at 7 μM, 0.36 ± 0.02 Hz at 10 μM; Hb9::Cre-Vglut2Δ/Δ cords: 0.20 ± 0.01 Hz

Figure 5. Silencing the output of glutamatergic Hb9::Cre-derived INs leads to reduced frequency of drug-evoked and neural-evoked locomotor-like activity. (A) Ventral root recordings of drug-evoked locomotor-like activity in control (upper traces) and Hb9::Cre-Vglut2Δ/Δ (lower traces) spinal cords at three concentrations of NMDA. All include 5-HT (8 μM). (B) Frequency of locomotor-like activity as a function of NMDA concentration on a constant background of 5-HT (8 μM) in control (blue) and Hb9::Cre-Vglut2Δ/Δ (green) spinal cords. Locomotor frequencies obtained in Hb9::Cre-Vglut2Δ/Δ cords were always lower than in control cords at all NMDA concentrations. Error bars represent ± SEM. *Indicates p < 0.001 and ** indicates p < 0.0001. (C) Circular plots of mean left-right (IL2-rL2) and flexor-extensor (rL2-rL5) coordination in control (blue) and Hb9::Cre-Vglut2Δ/Δ (green) spinal cords at 5 and 7 μM NMDA and 8 μM 5-HT. Each point corresponds to the mean vector value for each drug concentration (Left-right controls: 5 μM and 7 μM NMDA (N = 11); Left-right Hb9::Cre-Vglut2Δ/Δ cords: 5 μM NMDA (N = 8) and 7 μM NMDA (N = 13); Flexor-extensor Hb9::Cre-Vglut2Δ/Δ cords: 5 μM NMDA (N = 8) and 7 μM NMDA (N = 12)). Individual vector values were generated from 50 locomotor cycles in each spinal cord. The inner circle indicates significance level of p = 0.05. (D) Frequency of the descending fiber-evoked locomotor-like activity as function of stimulation amplitude. The maximal frequency obtained in Hb9::Cre-Vglut2Δ/Δ cords (green) was lower than in controls (blue) at stimulation strengths of 250 μA – 1 mA (controls: 0.35 ± 0.03 Hz (N = 12/13), 0.42 ± 0.03 Hz (N = 13/13), 0.46 ± 0.02 Hz (N = 12/13), 0.49 ± 0.02 Hz (N = 12/13); Hb9::Cre-Vglut2Δ/Δ cords: 0.32 ± 0.03 Hz (N = 11/16), 0.35 ± 0.02 Hz (N = 13/16), 0.36 ± 0.03 Hz (N = 13/16), 0.41 ± 0.03 Hz (N = 12/16) at 100 μA, 250 μA, 500 μA, and 1 mA, respectively). Error bars represent ± SEM. *Indicates p < 0.05 and **Indicates p < 0.005.
at 5 μM; 0.23 ± 0.006 Hz at 7 μM, 0.27 ± 0.005 Hz at 10 μM) (Fig. 5B). There was no significant difference in pattern generation as assessed by coordination of activity between the left and right sides of the cord (out of phase activity between right L2 and left L2) and in ipsilateral flexor-extensor coordination (out of phase activity between right L2 (flexor) and right L5 (extensor)) between control and Hb9::Cre-Vglut2Δ/Δ cords. Mean left-right phase values (Fig. 5C, left) in controls were 0.51 (N = 11) and 0.52 (N = 11) at 5 μM and 7 μM NMDA, respectively, and in Hb9::Cre-Vglut2Δ/Δ cords 0.47 (5 μM NMDA, N = 8) and 0.50 (7 μM NMDA, N = 13). Mean flexor-extensor phase values (Fig. 5C, right) in controls were 0.39 ± 0.02 Hz (N = 6/6), 0.44 ± 0.05 Hz (N = 6/6), 0.49 ± 0.06 Hz (N = 5/6); ChAT-Vglut2Δ/Δ cords: 0.35 ± 0.05 Hz (N = 6/6), 0.45 ± 0.04 Hz (N = 8/9), 0.53 ± 0.03 Hz (N = 9/9), 0.56 ± 0.02 Hz (N = 9/9) at 100 μA, 250 μA, 500 μA, and 1 mA, respectively. Error bars represent ± SEM.

In addition to drug-evoked locomotion, we also tested the effects of the loss of excitatory Hb9::Cre-derived INs on locomotion evoked by neural stimulation. It was possible to evoke locomotor-like activity by stimulating...
fibers descending from the brainstem in all control cords tested and in ~80% of the Hb9::Cre-Vglut2Δ/Δ cords (N = 13/16). The bursting frequency was reduced in Hb9::Cre-Vglut2Δ/Δ cords by 15–21% compared to controls at stimulation strengths of 250 μA–1 mA (p < 0.02) (Fig. 5D). Additionally, during electrical stimulation, the bursting was more difficult to discriminate in spinal cords from Hb9::Cre-Vglut2Δ/Δ mice.

Together, the lower drug-evoked and stimulus-evoked locomotor frequencies seen in spinal cords from Hb9::Cre-Vglut2Δ/Δ mice compared to controls suggest that excitatory spinal Hb9::Cre-derived INs play a significant role in rhythm generation.

**Loss of glutamatergic synaptic transmission in motor neurons does not affect the frequency of drug-induced or neural-evoked locomotor activity.** Hb9 is expressed by motor neurons and mammalian motor neurons co-release glutamate and acetylcholine from central collaterals. Since locomotion can be initiated by ventral root stimulation, we sought to determine if loss of Vglut2-mediated glutamatergic synaptic transmission in motor neurons would contribute to the locomotor phenotype observed in Hb9::Cre-Vglut2Δ/Δ spinal cords.

Similarly to our approach with Hb9::Cre mice, ChAT::Cre mice were used to generate ChAT-Vglut2Δ/Δ mice in which glutamatergic synaptic transmission was selectively lost in cholinergic neurons, including MNs. ChAT-Vglut2Δ/Δ mice are viable and show no clear phenotype; however, all experiments were performed at P0 for consistency. Locomotor-like activity was induced by bath application of NMDSA (5 μM–10 μM) and 5-HT (8 μM), and it was evoked in all control (N = 5/5) (Fig. 6A, upper traces) and ChAT-Vglut2Δ/Δ spinal cords (5 μM, N = 7/9; 7 μM and 10 μM NMDSA, N = 9/9) (Fig. 6A, lower traces). The locomotor frequency obtained in ChAT-Vglut2Δ/Δ cords was not significantly different from that of controls at any NMDSA concentration tested (p > 0.3) (controls: 0.27 ± 0.02 Hz at 5 μM, 0.32 ± 0.01 Hz at 7 μM, 0.40 ± 0.02 Hz at 10 μM; ChAT-Vglut2Δ/Δ cords: 0.27 ± 0.01 Hz at 5 μM, 0.32 ± 0.01 Hz at 7 μM, 0.37 ± 0.01 Hz at 10 μM) (Fig. 6B). Additionally, there was no significant difference in left-right (IL2–RL2) and flexor-extensor (RL2–RL5) phasing between control and ChAT-Vglut2Δ/Δ spinal cords (Watson-Williams’ Test, p > 0.4). Left-right and flexor-extensor activities were in alternation in both control and ChAT-Vglut2Δ/Δ cords. Mean left-right phase values (Fig. 6C, left) were 0.47 (5 μM, N = 6) and 0.47 (7 μM NMDSA, N = 6) for controls, and 0.50 (5 μM NMDSA, N = 8) and 0.51 (7 μM NMDSA, N = 9) for ChAT-Vglut2Δ/Δ cords. Mean flexor-extensor phase values (Fig. 6C, right) were 0.51 (5 μM, N = 4) and 0.43 (7 μM NMDSA, N = 4) for controls, and 0.51 (5 μM NMDSA, N = 8) and 0.47 (7 μM NMDSA, N = 9) for ChAT-Vglut2Δ/Δ cords.

To further verify if there were any deficits to the rhythm generating network due to the loss of glutamatergic transmission in motor neurons, we also used descending stimulation to evoke locomotion. Locomotor-like activity was evoked in all control (N = 6/6) and ChAT-Vglut2Δ/Δ (N = 9/9) spinal cords. In keeping with the results obtained from drug-induced locomotor studies, upon neural-evoked stimulation, no significant difference in locomotor frequency was observed between ChAT-Vglut2Δ/Δ and control cords at any of the stimulation intensities tested, 100 μA–1 mA (p > 0.3) (Fig. 6D).

Collectively, these data indicate that the reduction in locomotor frequency we observe in Hb9::Cre-Vglut2Δ/Δ cords is due to the loss of glutamatergic synaptic transmission in Hb9::Cre-derived INs and not in motor neurons.

**Discussion**

In this study, we have used the Hb9::Cre mouse line to identify Hb9::Cre-derived INs, target the excitatory subset of these neurons for removal from the spinal network, and investigate the consequences of their removal on motor output. Our findings provide insight into the involvement of these neurons in mammalian locomotor rhythm generation.

Hb9::Cre mice were generated with an analogous strategy to the Hb9-LacZ mice. However, a larger population of ventral as well as dorsal neurons is apparent in the Hb9::Cre-Rosa26-FP mouse. This is possibly due to the transient embryonic expression of Hb9 in progenitor cells and postmitotic neurons which is captured by the Cre-line. Here, we have collectively referred to the dorsal and ventral populations visualized in Hb9::Cre-Rosa26-FP mice as Hb9::Cre-derived INs.

Since in limbed animals the spinal locomotor networks are distributed rostro-caudally along the ventral lumbar spinal cord, the fact that the Hb9::Cre mouse also includes cells in the dorsal spinal cord does not hinder its use as a tool to study the role of excitatory Hb9::Cre-derived INs in locomotion. Moreover, despite the large number and laminar distribution of Hb9::Cre-derived INs, they comprise a reliably identifiable population of interneurons. The same neurons are visualized by reporter expression in all Rosa26 lines throughout the lumbar spinal cord. Additionally, as demonstrated by Vglut2 mRNA in situ, this same visualized population is manipulated in the Hb9::Cre-Vglut2Δ/Δ mice.

Therefore, by using Cre-Lox recombination, we were able to reliably manipulate a group of excitatory INs which does not correspond to any of the known major excitatory ipsilaterally-projecting classes of neurons. In particular, excitatory Hb9::Cre-derived INs delineate a group of neurons that is distinct from the only other excitatory neuronal population that has been shown to play a role in mammalian locomotor rhythm generation, the Shox2 non-V2a neurons.

The cardinal feature that should characterize rhythm-generating neurons is that their selective manipulation should have a direct impact on locomotor frequency. Their activation should be able to initiate locomotor rhythm and/or change the frequency of the ongoing rhythm whereas a selective reduction in their number should reduce the frequency of the ongoing locomotor rhythm. The most conclusive test to determine the effects of a given group of neurons on locomotor activity is achieved by an acute activation or inactivation of these neurons. However, the combination of channelrhodopsin (or other cell activator or inactivator) mouse lines with Hb9::Cre mice would lead to expression in both inhibitory and excitatory Hb9::Cre-derived INs as well as in MNs, thus confounding results. The inclusion of MNs in the population makes optogenetic or chemogenetic perturbation
experiments impossible to perform. Therefore, we instead pursued the functional removal of glutamatergic Hb9::Cre-derived INs from the network by selectively deleting Vglut2 from the excitatory Hb9::Cre-derived population. By silencing the output of glutamatergic Hb9::Cre-derived INs and studying motor output in an isolated spinal cord preparation, we have shown that excitatory Hb9::Cre-derived INs are likely part of the locomotor rhythm generator, as demonstrated by the reduced locomotor frequency. In contrast, silencing the output of Vglut2-expressing Hb9::Cre-derived INs had no effect on patterning, including left-right and flexor-extensor alternations, unlike what has been described for the manipulation of excitatory V2a, V0 or V3 neurons.12,14,16,50 We acknowledge, however, that distinguishing between a role in tonic drive to the rhythm generator and a role in rhythm generation per se is difficult and nearly impossible experimentally, and therefore, we cannot exclude that this decrease in frequency might be partly due to a reduction in tonic drive to the rhythm generating core.

The fact that some of the spinal cords from Hb9::Cre-Vglut2Δ/Δ mice did not respond to descending fiber stimulation may suggest that glutamatergic Hb9::Cre-derived INs play a role in mediating the initiation of locomotion. Thus, it is possible that the descending command system which initiates locomotion may target glutamatergic Hb9::Cre-derived INs and, due to the functional inactivation of these glutamatergic interneurons in Hb9::Cre-Vglut2Δ/Δ mice, locomotion is less readily evoked by stimulation of the descending fibers. With the genetic tools at hand, it is not possible to determine whether the canonical Hb9 INs are partly or solely accountable for the locomotor phenotype we observe in Hb9::Cre-Vglut2Δ/Δ spinal cords. However, canonical Hb9 INs make up less than 1% of the Hb9::Cre-derived INs, and 33% of these Hb9::Cre-derived INs are excitatory. Therefore, the canonical Hb9 INs would be a negligible portion of the excitatory Hb9::Cre-derived INs (<3%). Accordingly, in Hb9::Cre-Vglut2Δ/Δ cords, the small number of canonical Hb9 neurons as compared to the total number of excitatory Hb9::Cre-derived INs, makes it less likely that the canonical Hb9 interneurons alone are responsible for the observed phenotype. It should be emphasized that, similarly to what was concluded in studies on Shox2 neurons,28 we believe that it is unlikely that excitatory Hb9::Cre-derived INs are the sole rhythm-generating neurons in the mammalian locomotor network. In the absence of excitatory Hb9::Cre-derived INs, locomotor frequency is reduced but locomotor rhythm is not abolished. This is seen more clearly when compared to experiments where all excitatory neurons between molecularly distinct groups of excitatory neurons may contribute to rhythm generation. A distribution of rhythm generation between molecularly distinct groups of excitatory neurons is also seen in young zebrafish where excitatory commissural neurons51-53 and V2a domain are involved in rhythm generation54-56. However, the corresponding Shox2 INs and excitatory Hb9::Cre-derived INs have not yet been identified in the zebrafish.

Our classification of neurons as Hb9::Cre-derived INs does not sprout from the initial progenitor domain-defined classes of interneurons. Instead, Hb9::Cre-derived INs, here, are those identified in the Hb9::Cre mouse line49. We have shown that excitatory Hb9::Cre-derived INs are a heterogeneous group of neurons that span several of the transcription factor-defined interneuron classes. Similarly, studies on Shox2 INs have shown that even within Shox2 non-V2a neurons, rhythm generation may be distributed amongst neurons derived from several progenitor domains. Collectively, these data suggest that glutamatergic neurons spanning several of the known molecularly defined groups of neurons are responsible for rhythm generation. Therefore, it is plausible that, in the same way as new markers for acetylcholinergic spinal neurons57,58 and inhibitory neurons59,60 were identified, a comprehensive transcriptome screening to define a finer-grained molecular code of spinal glutamatergic neurons may define new markers for excitatory neurons that may help to clarify the identity of the functional subgroups involved in mammalian locomotor rhythm generation.

Experimental Procedures. Mouse lines. All experimental procedures followed the guidelines of the Animal Welfare Agency and were approved by the local ethical committee, Stockholms Norra Djurförsökningsnämnd. The following transgenic lines were used: Hb9::Cre (B6;129S-Mnx1tm4(cre)Tmj/J) mice obtained from the Jackson Laboratory see ref. 40; Vglut2Flox/Flox and Vglut2Δ/Δ mice kindly provided by Dr. H.U. Zeilhofer63; Glyt2-GFP mice kindly provided by Drs. T Hnasko and R. Palminter45; Vglut2-GFP mice generated in our lab by Dr. Borgius using BAC recombination with an analogous strategy to the one described previously (Supplementary Figure 1); Hb9-GFP (B6.Cg-Tg(Hbx9-GFP)1Tmj/J) mice obtained from the Jackson Laboratory; GlyT2-GFP mice kindly provided by Dr. H. U. Zeilhofer63; Gad67-GFP mice provided by Dr. Y. Yanagawa49; ChAT::Cre (B6;129S6-Chattm2(cre)Low/J) mice, Rosa26-YFP (Rosa26FlsostmSTOP-YFP) and Rosa26-tdTomato (Rosa26FlsostmSTOP-TdTomato) mice obtained from the Jackson Laboratory.

For conditional deletion of Vglut2, Vglut2Δ/Δ reporter mice with loxp sites flanking exon 2 of the Slc17a6 gene, which encodes for Vglut2, were used (see ref. 17). To avoid germ-line recombination we first crossed either Hb9::Cre or ChAT::Cre with Vglut2Δ/Δ mice to obtain the double transgenic lines Hb9::Cre;Vglut2Δ/Δ or ChAT::Cre;Vglut2Δ/Δ. These double transgenic lines were then crossed with the homozygous Vglut2Δ/Δ reporter Rosa26-YFP mice to generate Hb9::Cre;Vglut2Δ/Δ or ChAT::Cre;Vglut2Δ/Δ mice (see ref. 28), with conditional removal of Vglut2 and conditional expression of YFP.

For neurotransmitter phenotyping Vglut2-GFP, Gad67-GFP or Glyt2-GFP mice were crossed with the reporter line Rosa26-tdTomato to obtain the double transgenic lines Vglut2-GFP;Rosa26-tdTomato, Gad67-GFP;Rosa26-tdTomato or Glyt2-GFP;Rosa26-tdTomato. These double transgenic lines were then crossed with Hb9::Cre mice to generate the triple transgenic lines Hb9::Cre;Rosa26-tdTomato;Vglut2-GFP, Hb9::Cre;Rosa26-tdTomato;Gad67-GFP, or Hb9::Cre;Rosa26-tdTomato;Glyt2-GFP.

In Situ Hybridization, Immunohistochemistry and Cell Counts. Spinal cords from embryonic mice at stage E11.5 and lumbar spinal cords from newborn mice at P0 were immersion fixed in 4% paraformaldehyde (2 hours at room temperature for immunohistochemistry or 24 hours at 4 °C for in situ hybridization), cryopreserved in 30% sucrose overnight, and stored at −80 °C. Serial transverse sections of embryonic spinal cords and newborn lumbar spinal cords were cryostat sectioned (20 μm) and mounted on Menzel-Glaser SuperFrost slides. All slides
were stored at –80°C. Sections were incubated for 24 hours with one or several of the following primary antibodies: rabbit anti-Shox2 #860 (1:32,000, generated against the peptide CKTTSKNSIHADLR), sheep anti-Chx10 (1:400, Chemicon), mouse anti-Isl1 (40.2D6 and 39.4D5) (1:250, DSHB), rabbit anti-Hb9 (1:16000, DSHB). The specificity of these antibodies has been evaluated and described in previous publications (see refs 12, 28 and 35). Secondary antibodies were obtained from Jackson ImmunoResearch or Invitrogen, used at 1:500 and incubated for 2 hours at room temperature.

Combined fluorescent in situ hybridization and immunofluorescence labeling was performed as previously described using an antisense digoxigenin (DIG)-labeled RNA probe for vesicular glutamate transporter 2 (Vglut2) that spans base pairs 540 to 983 (produced by Dr. L. Borgius). Sections were blocked with 0.5% Blocking reagent (PerkinElmer) in TNT (0.1 M Tris-Hcl pH7.5, 0.15 M NaCl, 0.1% (wg/vol) Tween20) and incubated with sheep anti-DIG peroxidase (1:2000, Roche Diagnostics). Signal was detected by cyanine 3 (Cy3) amplification (1:50, PerkinElmer).

Slides were mounted in Vectashield reagent (Vector Laboratories), and images were scanned on a LSM510 Confocal Microscope (Zeiss Microsystems) using 10x and 20x objectives. Multiple channels were scanned sequentially to prevent fluorescence bleed through and false-positive signals. A contrast enhancement and noise reduction filter were applied in ImageJ for publication images.

Counts were done manually using a cell-counter plug-in in ImageJ, in 4–11 non-adjacent sections per mice (N = 2–4) in each condition. Cellular counts per hemi-section were averaged per individual animal, and a grand-mean ± standard error of the mean (SEM) was calculated across animals to produce percentage of cell per hemi-section bar-graphs.

**In Vitro Recording of Locomotor-Like Activity.** Spinal cords from newborn mice at P0 were dissected in ice-cold low Ca2+ Ringer’s solution in mM: (111 NaCl, 3 KCl, 11 glucose, 25 NaHCO3, 3.7 MgSO4, 1.0 K2HPO4 and 0.25 CaCl2) pH adjusted to 7.4 with 95% O2/5% CO2. All preparations were transferred to a recording chamber and perfused with normal Ringer’s solution in mM; (111 NaCl, 3 KCl, 11 glucose, 25 NaHCO3, 1.3 MgSO4, 1.1 KH2PO4, 2.5 CaCl2, pH 7.4, aerated with 95% O2/5% CO2) at a flow rate of 4–5 ml/min. All recordings were performed at room temperature. Locomotor-like activity was either induced by bath application of N-methyl-D-aspartate (NMDA; 5–10 μM) and serotonin (5-HT; 5–8 μM) or evoked by electric stimulation of descending pathways86. Briefly, the electrical stimulation protocol entails a large suction electrode (approximately 100 μm in tip diameter) used to deliver large amplitude (0.1–1 mA), long duration (10 ms) and low frequency (2 Hz) stimuli at the midline over the C1–C2 segments. The total duration of each train of stimuli was 60 seconds.

Recordings were obtained using glass suction electrodes attached to lumbar ventral roots (VRs) L2 or L5 on the left and right sides of the cord. VR signals were AC recorded and band-pass filtered from 100 to 1000 Hz (gain 5–10,000), digitized, and acquired with pCLAMP software (version 10; Molecular Devices).

**Locomotor analysis.** Locomotor frequency (Hz) (defined as the reciprocal of locomotor cycles) was calculated from 3–5 min of activity, taken at least 15 min after the initial burst of drug-induced activity, when locomotor-like activity had stabilized. Locomotor-like activity was analysed using rectified, smoothed (time constant of 0.2 s) and filtered AC signals of VR activity in Spike2 (Cambridge Electronic Design). Statistical significance was determined using two-way ANOVA and Sidak’s multiple comparisons test or multiple T test using the Holm-Sidak method, with alpha = 0.005% (p < 0.05). All p-values were adjusted to account for multiple comparisons. Data are expressed as mean ± standard error of the mean (SEM).

For the analysis of left-right and flexor-extensor coordination, 50 random locomotor cycles were selected. Left (l) L2 VR was chosen as the reference trace while right (r) L2 and rL5 VRs were used as test traces. Circular statistics were used to determine the phase relationship between ipsilateral and contralateral VRs during locomotor-like rhythmic activity as previously described13,17,20. The onset of a locomotor burst in the reference trace corresponds to the phase value of 0 in the circular plot and the onset of the next burst corresponds to a phase value of 1. For test traces, a phase value of 0.5 corresponds to alternation while a phase value of 1 (or 0) corresponds to synchrony. The mean timing of the 50 phase values was calculated as a vector in the circular plot where the resulting vector length (r) reflects the concentration of phase values around the mean. Rayleigh’s test66 was used to determine whether r values reached statistical significance (p < 0.05). Points falling inside the inner circle are not significant while those outside the inner circle are significant. Data are expressed as grand mean for each drug concentration. Watson-Williams test67 was used to determine significant differences amongst conditions (p < 0.05).

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K.J.D. and O.K. conceived the electrophysiological study. V.C., K.J.D. and O.K. conceived the immunohistochemical study. V.C. and K.J.D. performed immunohistochemical investigations, in vitro recordings and analyzed data. L.B. generated the Vglut2 Flox/Flox mice. V.C. and O.K. wrote the paper with contributions from all authors. O.K. supervised all aspects of the work.

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Additional Information

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