Heterogeneous Electrotonic Coupling and Synchronization of Rhythmic Bursting Activity in Mouse Hb9 Interneurons

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INTRODUCTION

A discrete population of interneurons, genetically defined by expression of the homeodomain protein Hb9, has been identified in the mouse spinal cord (Hinckley et al. 2005; Wilson et al. 2005). Hb9 interneurons are glutamatergic and have homogeneous electrophysiological properties, most notably postinhibitory rebound (PIR) (Wilson et al. 2005). Furthermore, Hb9 interneurons are conditional oscillators as revealed by their response to neurotransmitters that induce locomotor output in the isolated spinal cord, suggestive of their involvement in rhythm generation (Jiang et al. 1999; Wilson et al. 2005). In some Hb9 interneurons, oscillations were tens of millivolts in amplitude with frequencies similar to in vitro locomotor frequencies. In Hb9 interneurons with smaller-amplitude oscillations, oscillation frequency was voltage independent, raising the possibility that these interneurons are electrotonically coupled (Sillar and Simmers 1994; Wilson et al. 2005). Interestingly, nearby non-Hb9 interneurons demonstrated voltage-independent spikelets, also characteristic of electrotonic coupling (Wilson et al. 2005). These findings suggest either homogeneous (e.g., between Hb9 interneurons) or heterogeneous (e.g., between Hb9 and non-Hb9 neurons) electrotonic coupling. Recently evidence was presented supporting homogeneous electrotonic coupling between Hb9 interneurons (Hinckley and Ziskind-Conhaim 2006).

Electrotonic coupling can contribute to the generation of rhythmic network activity in diverse systems. In Tritonia, interneurons involved in swimming activity are electrotonically coupled (Getting et al. 1980). In the stomatogastric ganglion of the lobster, gap junctions connect three different interneuronal populations involved in rhythm generation (Eisen and Marder 1982). Electrotonic coupling of brain stem neurons may generate or modulate the respiratory rhythm in mammals (Rekling et al. 2000). Thus electrotonic coupling can be present both within and between populations of rhythm-generating neurons.

Ultrastructural studies have demonstrated the prevalence of gap junctions in the mature mammalian spinal cord (Rash et al. 1996, 2000). Electrotonic coupling has been implicated in motoneuron bursting in neonatal rats (Chang et al. 1999; Tresch and Kiehn 2000; Walton and Navarrete 1991), and network oscillatory activity in both the dorsal horn (Asghar et al. 2005) and intermediolateral cell column (Logan et al. 1996). Spontaneous rhythmic episodes in the embryonic rat spinal cord that persist in the absence of chemical transmission are facilitated, in part, by electrotonic coupling (Ren et al. 2005). In the postnatal rat spinal cord, locomotor-like activity induced by neurotransmitters, potassium channel blockers, or high extracellular potassium concentrations persists in the absence of fast chemical synaptic transmission, indicating electrotonic coupling may contribute to motor rhythm generation (Kiehn and Tresch 2002; Taccola and Nistri 2004).

In this study, the hypothesis that Hb9 interneurons, which may be involved in locomotor rhythm generation, are involved in an electrotonically coupled network is investigated. We use a combination of techniques to present evidence that although Hb9 interneurons burst in phase with each other and are synchronous with rhythmic ventral root output, they are not electrotonically coupled with each other. Rather they are cou-
plied to other non-Hb9 interneurons. This heterogeneous electrotonic coupling may be important to ensure that the network can produce the range of stable rhythms necessary for locomotion (Manor et al. 1997; Soto-Trevino et al. 2005).

**METHODS**

All procedures and experiments were approved by the Dalhousie University Committee for Laboratory Animals, which conforms to the standards set by the Canadian Council of Animal Care and the Australian National University Animal Experimentation Ethics Committee. All experiments involved Hb9::eGFP transgenic mice (Wichterle et al. 2002). Postnatal mice [postnatal day 3 (P3) to P12] were deeply anesthetized with ketamine (100 mg/kg ip) or trichloroethylene (Avertin, 400 mg/kg), a laminectomy was performed, and their spinal cords were removed. For experiments using spinal cord slices (P8–P12), the cord was set in agar and transverse slices (200–300 μm) were cut with a vibrating microtome (Vibratome 3000; Vibratome, St. Louis, MO) or Microslicer DTK-01, Dosaka) as previously described (Wilson et al. 2005). For the in vitro spinal cord preparation (P3–P6), suction electrodes were attached to bilateral dorsal lumbar ventral roots (Jiang et al. 1999). Aesthetically transverse fluid (ACSF) was superinfused during experimentation, and for the single-cell patch-clamp experiments contained (in mM) 127 NaCl, 1.9 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgCl2, 26 NaHCO3, and 10 μg of glucose. For the dual cell experiments, the solution contained 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 25 NaHCO3, and 10 μg of glucose. Single-cell patch-clamp recordings were undertaken at room temperature, whereas the dual recordings and the multiphoton imaging data were obtained at 30–32°C.

Patch-clamp electrodes (5–9 MΩ) contained (in mM) 130 K-gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 1 CaCl2, and 4 Mg-ATP and 70 μM Alexa 594 (Invitrogen Burlington, Ontario, Canada). For the dual cell recordings, the electrode solution contained (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 4 Mg-ATP, 0.3 GTP, and 0.2–0.5% bicuculline. Whole cell patch-clamp recordings were obtained using differential interference contrast optics under infrared illumination with a Multi-Clamp 700A amplifier, a Digidata 1322A or ITC18 A/D converter, and a G4 Macintosh computer running AxoGraph 4.9 (Molecular Devices, Union City, CA) or Igor Pro (Wavemetrics, Portland, OR) software. The following pharmacological agents were bath applied at the indicated concentrations: TEA, 4-aminopyridine (4-AP), BaCl2, carbenoxolone, bicuculline, strychnine (Sigma-Aldrich Canada, Oakville, Ontario, CA), t-APS, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[1]quinoxaline (NBQX) (Tocris Bioscience) and tetrodotoxin (TTX, Alomone Labs, Jerusalem, Israel).

**Immunohistochemistry**

For immunohistochemical experiments (see Wilson et al. 2005), Hb9::GFP mice were crossed with Hb9::IRES-BAC::lacZ+/− mice, and the double-positive offspring of specific ages were intracardially perfused with 4% paraformaldehyde. A laminectomy was performed, and their spinal cords were removed and postfixed over night in 4% paraformaldehyde before cryoprotection in 30% sucrose. Spinal cord sections (50 μm) were cut on a freezing microtome and incubated with primary antibodies [rabbit (1:500) or sheep (1:500) anti-GFP and mouse anti-β-galactosidase (β-Gal; 1:2000) Chemicon, Temecula, CA] at 4°C for 24 h. Sections were thrice washed in PBS before a 2-h incubation with appropriate secondary antibodies conjugated to Alexa 488 or Alexa 555 (1:500–1,000, Invitrogen). Confocal micrographs were acquired with a Zeiss LSM 510 Meta laser scanning microscope equipped with an Axiovert 200 m inverted microscope, oil immersion objectives (×25 to ×63) and Arsen (488 nm) and HeNe (543 nm) lasers. Z-stacks were collected at <1 μm, and projection images were compiled from 9 to 20 images and imported into Adobe Photoshop (Adobe Systems, San Jose, CA) for illustration purposes.

**Identification of Hb9 interneurons**

Due to the “ectopic” expression of GFP in postnatal Hb9::eGFP mice (Wilson et al. 2005), it is necessary to have criteria by which GFP positive neurons may be identified as Hb9 interneurons. The spinal cord slice experiments were performed in slices from P8–P12 “motor functionally mature” mice, defined as mice at a developmental stage such that they can walk and wade with their body weight supported (Jiang et al. 1999). At this stage, putative Hb9 interneurons can be identified as those GFP-positive neurons in medial lamina VIII abutting the ventral commissure with characteristic morphology (small, round somata, few neurites). The majority of these neurons formed tightly clustered pairs, although single interneurons that matched the above morphological criteria were also evident (Wilson et al. 2005) (Fig. 1D). Both these single interneurons and the tightly clustered pairs were confirmed to be Hb9 interneurons by using whole cell patch-clamp recordings as Hb9 interneurons demonstrate very high-input resistance, low whole cell capacitance, and strong post-hibernationary rebound not associated with an apparent Ih-like current (Wilson et al. 2005). At this age, all interneurons that met the strict morphological criteria also met the electrophysiological criteria. That is, the specificity for using the morphological criteria alone to determine Hb9 interneuron identity is very high. All electrophysiological data presented here were obtained from neurons that met both the morphological and electrophysiological criteria.

The imaging studies in spinal cord slices were carried out at similar postnatal ages, and therefore Hb9 interneurons were included in the study if they met the morphological criteria listed in the preceding text, including, importantly, formation of tightly clustered pairs (e.g., Figs. 1, C and D, and 3B). Thus Hb9 interneurons were readily identifiable in late postnatal mice of a developmental stage such that they could walk (i.e., 2nd postnatal week).

In contrast, the imaging studies in the isolated spinal cord were performed in younger mice (P3–P6). We therefore investigated whether we could accurately ascertain the identity of Hb9 interneurons in this preparation. Using double transgenic animals in which a nuclear lacZ is knocked into an Hb9 locus (Arber et al. 1999; Wilson et al. 2005), we investigated the relation of Hb9-positive to Hb9-negative GFP-positive interneurons in lamina VIII. At younger ages (P2–4), GFP-positive interneurons are densely located in median lamina VIII (Fig. 1, A–C), and although many of them were Hb9 positive, almost half of these neurons were Hb9 negative (Fig. 1A, arrow). The Hb9 interneurons at these ages have heterogeneous morphology with somata size and shape varying considerably (Fig. 1B). However, Hb9 interneurons with small round somata in tightly clustered pairs could still be discerned (Fig. 1C, arrowhead) within the total population of Hb9 interneurons and surrounding GFP-positive (Hb9-negative) interneurons. This contrasts with the older animals (P8–12), in which most of the GFP-positive neurons clustered in this area are Hb9 positive and form pairs (Fig. 1D, arrowhead). Thus by their distinct location and morphology, these pairs can be consistently studied throughout postnatal development.

In the isolated spinal cord, these morphologically distinct neurons were found 130–180 μm from the ventral surface of the spinal cord, where they formed two distinct bilateral columns running in the rostrocaudal direction. Their identity was confirmed in longitudinal fixed sections (i.e., similar to the 2 photon imaging plane) where tightly clustered pairs of Hb9 interneurons could readily be discerned (Figs. 1, E and F).

**Calcium imaging**

The techniques for the use of two photon excitation fluorescence microscopy of calcium-sensitive dyes in GFP-positive neurons have
recently been described (Wilson et al. 2007). Briefly, cells were loaded with Fluo-3 AM (Invitrogen) using a technique similar to that described by Stosiek et al. (2003). An initial 1 mM stock solution of Fluo-3 AM dissolved in dimethyl sulfoxide and 20% pluronic acid was diluted 1:10 with ACSF for a final loading concentration of 100 μM. A filled micropipette was positioned close to Hb9 interneurons and pressure applied (100–200 mmHg for an average of 2 min). Beginning 45 min later, interneurons were visualized with an upright Zeiss Axiovert II microscope equipped with a LSM510 NLO Meta scan head (Carl Zeiss, Toronto, Ontario, Canada) and a tunable near infrared laser (Chameleon XR, Coherent, Santa Clara, CA). Reflected light was directed to nondescanned detectors (R6357, Hamamatsu Photonics KK, Shizuoka, Japan).

Hb9 interneurons could be readily identified by excitation of GFP at 960 nm when emissions were collected through a 465- to 495-nm filter. Fluo-3-AM was excited at 800 nm, and emissions were collected through a 500- to 550-nm filter. There was minimal excitation of GFP at 800 nm at the same laser power that excited Fluo-3-AM, evidenced by the low signal collected between 465 and 495 nm. Time series of fluorescent images were collected with the following parameters: 512 × 512 pixel images, digital zoom four to eight times with ×40 objective (N.A. 0.8), 800 frames, 200–400 ms/frame, 0.64 μs pixel dwell time, laser power <50 mW. Bidirectional scanning was used to increase scan speed, and scanners were always calibrated in the XY plane before each acquisition. In the experiments using the isolated spinal cord preparation, ventral root electrophysiograms were captured (Digidata 1322A and Axoscope software, Molecular Devices) along with a TTL pulse emitted from the scanhead at the start of every frame so as to synchronize the ventral root recordings with the calcium imaging traces.

Data analysis

Electrophysiological data were analyzed using Clampfit 9.0 software (Molecular Devices) and Microsoft Excel or Igor Pro (WaveMetrics). All values are expressed as the means ± SD. The coefficient of variation (CV) is 100 times the SD divided by the mean. Spikelets were recognized by their characteristic waveform, small amplitude (<10 mV) and voltage independence (Bennett 1977).

Synchronicity of events [excitatory postsynaptic potentials (EPSPs), inhibitory PSPs (IPSPs), or spikelets] was determined by cross-correlation analysis using a bin size of 5 ms for 60-s epochs. EPSPs, IPSPs, or spikelets were detected as discrete events in time by marking their peaks. The 5-ms time window was chosen as this was empirically determined to be the optimal template length for identifying discrete events with the highest accuracy while still being able to discriminate between closely spaced events. In addition, to determine whether the events in each of two neurons were related and not simply occurring according to a random process, we calculated the “synchrony percentage,” i.e., the proportion of events that were synchronous. If the events were independent, not related and distributed according to a Poisson process, then the probability function \( F(t) \) can be calculated by

\[
F(t) = 1 - e^{-(\lambda_1 + \lambda_2)t}
\]

where \( \lambda_1 \) and \( \lambda_2 \) are the frequencies of the events in neurons 1 and 2, and \( t \) is the time window defined for synchronous events (Bornstein 1978). We defined events in Hb9 interneurons as synchronous when they occurred within 5 ms of each other. In these neurons, the typical spontaneous frequencies of these events were in the range of 10 Hz; and thus the maximum synchrony percentage due to chance using \( t = 5 \) ms would be \( F = 1 - \exp(-0.1) \sim 10\% \). Therefore any synchrony percentage >10% was taken to indicate that the events were related and not random.

Calcium transients were recorded in mean pixel intensity in a defined region of interest over time and expressed as the

![Identification of Hb9 interneurons](image-url)
percentage change in fluorescence divided by the baseline fluorescence (delta \( F/F \)). These data were analyzed in Excel and figures compiled using SigmaPlot 9.

The data obtained from the isolated spinal cords were analyzed using custom software developed at the Spinal Cord Research Centre, University of Manitoba (SCRC Data Capture and Analysis Software: http://www.ssrc.umanitoba.ca/doc/) on Mac OS X. This analysis package was modified to allow the alignment of the calcium imaging waveform with the TTL pulses and hence with ventral root recordings. Because of the time taken to capture each frame (200–400 ms), we could not use a window of 5 ms to define synchrony. Instead, the temporal relationship between the calcium activity in a given neuron and the ventral root output was quantified using methods similar to those defined in (Kriellaars et al. 1994). After normalizing the waveforms for step cycle durations, the phase relationships between the onsets of activity in pairs of waveforms (calcium signals and ventral root recordings) were calculated. The phase relationships were then expressed as degrees and plotted on polar plots, where 0 represents in phase and 180 represents antiphase relations.

RESULTS

TEA and 4-AP induced rhythmic activity in HB9 interneurons

We previously demonstrated that HB9 interneurons are conditional endogenous bursters; that is, in TTX, the membrane potential of HB9 interneurons in slice undergoes large amplitude oscillations in response to the same chemical stimuli that evoke fictive locomotion in the isolated mouse spinal cord (Wilson et al. 2005). To study electrotonic coupling involving HB9 neurons (Wilson et al. 2005), we selected a model in which rhythmic motor output can be evoked by raising the excitability (Bracci et al. 1998; Taccola and Nistri 2004; Zangger 1981). The first step was to determine whether HB9 interneurons also become rhythmically active in response to this paradigm. Therefore potassium channels were blocked by bath applying TEA (6–30 mM) and 4-AP (1–4 mM) to spinal cord slices, and whole cell patch-clamp recordings were obtained from HB9 interneurons. The presence of TEA and 4-AP had minimal effect on the resting membrane potential of HB9 interneurons (+1.1 ± 0.7 mV, \( n = 9 \)); however, as expected, the action potential duration, measured at threshold, increased from 3.5 ± 1 to 55.5 ± 26 ms (averages of 10 action potentials in each of 3 neurons; supplementary Fig. 1). TEA and 4-AP induced an increase in the frequency and amplitude of spontaneous synaptic events in all HB9 interneurons (\( n = 9 \); Fig. 2A, i and ii). At a baseline of –50 mV, these events could reach threshold for action potential generation eliciting a burst (Fig. 2Aii). In only some cells were the bursts regular enough to analyze, with mean burst durations of 1.1 ± 0.3 s, and burst frequencies of 0.16 ± 0.07 Hz (CV = 47.8; \( n = 4 \)). These data indicate that HB9 interneurons in slice, in addition to being rhythmically active in response to the same chemical stimuli which evoke fictive locomotion in the isolated mouse spinal cord (Wilson et al. 2005), become rhythmically active and burst in response to other stimuli which can evoke rhythmic motor output: potassium channel blockers (Taccola and Nistri 2004).

To investigate the involvement of chemical synaptic transmission in generating these bursts, neurotransmitter receptor antagonists were applied in the presence of TEA and 4-AP. Fast inhibitory transmission was blocked with bicuculline (10 mM) and strychnine (1 mM), which can evoke rhythmic motor output: potassium channel blockers (Taccola and Nistri 2004; Zangger 1981). The first step was to determine whether Hb9 interneurons also become rhythmically active in response to this paradigm. Therefore potassium channels were blocked by bath applying TEA (6–30 mM) and 4-AP (1–4 mM) to spinal cord slices, and whole cell patch-clamp recordings were obtained from HB9 interneurons. The presence of TEA and 4-AP had minimal effect on the resting membrane potential of HB9 interneurons (+1.1 ± 0.7 mV, \( n = 9 \)); however, as expected, the action potential duration, measured at threshold, increased from 3.5 ± 1 to 55.5 ± 26 ms (averages of 10 action potentials in each of 3 neurons; supplementary Fig. 1). TEA and 4-AP induced an increase in the frequency and amplitude of spontaneous synaptic events in all HB9 interneurons (\( n = 9 \); Fig. 2A, i and ii). At a baseline of –50 mV, these events could reach threshold for action potential generation eliciting a burst (Fig. 2Aii). In only some cells were the bursts regular enough to analyze, with mean burst durations of 1.1 ± 0.3 s, and burst frequencies of 0.16 ± 0.07 Hz (CV = 47.8; \( n = 4 \)). These data indicate that HB9 interneurons in slice, in addition to being rhythmically active in response to the same chemical stimuli which evoke fictive locomotion in the isolated mouse spinal cord (Wilson et al. 2005), become rhythmically active and burst in response to other stimuli which can evoke rhythmic motor output: potassium channel blockers (Taccola and Nistri 2004).

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these data indicate that inhibitory synaptic input can modulate bursting through prolongation of the interburst interval as well as by reducing spiking during the burst. However, inhibitory transmission is clearly not necessary in the production of these bursts.

Next, to determine the role of excitatory synaptic transmission in the bursting activity, the AMPA/kainate receptor antagonist NBQX (5 μM, n = 7) and/or the NMDA receptor antagonist AP5 (10 μM, n = 4) were applied to block fast AMPA/kainate and NMDA receptor-mediated glutamatergic transmission, respectively. In experiments in which NBQX, bicuculline, and strychnine were added to the slice in the presence of TEA/4-AP, the burst duration was longer (2.1 ± 0.5 s, P < 0.05, n = 6), and the burst frequency slower (0.10 ± 0.03 Hz, P < 0.05; CV: 27.9) compared with 4-AP and TEA alone. The bursting activity remained following subsequent addition of AP5 (n = 4). AP5 had little additive effect to the NBQX in that there was no further change in burst frequency (0.09 ± 0.02 compared with 0.1 ± 0.03 Hz, P > 0.05) and minimal change in burst duration (2.4 ± 0.5 s compared with 2.1 ± 0.4 s, P < 0.05, Fig. 2Cii). In all conditions, the burst frequency was independent of membrane voltage over the range tested (−50 to −75 mV). These data indicate that glutamatergic transmission may increase the excitability of the Hb9 interneurons but does not play a role in the genesis of these oscillations. Further, the addition of antagonists to fast chemical synaptic transmission in the presence of potassium channel blockers leads to stable and persistent rhythmic activity in Hb9 interneurons.

### Evidence for a role of electrotonic coupling in Hb9 interneuron rhythmicity

Given that neither excitatory nor inhibitory synaptic transmission is necessary for producing oscillations in Hb9 interneurons in response to TEA and 4-AP, we proceeded to analyze the spiking data for evidence of electrotonic coupling. Note that even though dye coupling was not seen with either Alexa 594 or biocytin, this does not preclude the presence of gap junctions (Logan et al. 1996). The bursting in TEA and 4-AP consisted of an initial spike followed immediately by low-amplitude spikelets (Fig. 2Aii, see following text). These spikelets were voltage independent (Fig. 2D). On average there were 1.1 ± 0.1 action potentials per burst, and 1.5 ± 1.2 spikelet/burst (Fig. 2B). Voltage-independent spikelets with comparable waveforms have been shown to be the correlates of electrotonic coupling, arising from action potentials produced in electrotonically coupled neurons (Bennett 1977; Fuentesbalba et al. 2004; Hughes et al. 2002; Perez Velazquez and Carlen 2000). In the presence of bicuculline and strychnine, the average number of action potentials (3.2 ± 1.2 AP/burst, Fig. 2B) was greater than that seen in 4-AP and TEA alone. Occasional spikelets could be observed within a burst at −50 mV (0.9 ± 1.1 spikelet/burst). Injection of hyperpolarizing current blocked most action potentials and revealed underlying spikelets that formed bursts of similar duration and frequency (Fig. 2A and D). In the presence of NBQX, the number of action potentials (2.7 ± 1.7 spike/burst, P < 0.05) was greater than that in TEA and 4-AP alone as were the number of spikelets (5.4 ± 1.5, P < 0.05, n = 7). The total number of spikes plus spikelets increased progressively with application of each antagonist (TEA +4-AP: 2.5 ± 1.2; + bicuculline + strychnine: 4.1 ± 1.8; + NBQX; 7.7 ± 2.2; + AP5: 9.1 ± 1.4, P < 0.05, Fig. 2B). The increase in spiking activity may result from an increase in input resistance, a reduction in inhibitory input, small differences in the resting membrane potentials between the cells, increased excitability in the network, or a combination of these factors.

To determine whether sodium channels were involved in the bursting activity, we next applied TTX (1 μM) to the cells in the presence of TEA, 4-AP, bicuculline, strychnine, NBQX, and AP5. TTX initially shortened the bursts (data not shown) and subsequently blocked all oscillatory activity (n = 4; Fig. 2Ciii). TTX did not eliminate the ability of these neurons to undergo endogenous rhythmic membrane potential oscillations, as oscillations in membrane potential could be evoked by subsequent addition of barium (1 mM; n = 2, supplemental Fig. 2). This may result from an additional block of potassium conductances and/or from an increase in current mediated by voltage-gated calcium channels. The initial shortening of the bursts after TTX application could result from reduced calcium influx secondary to a reduction in the sodium spikes or by block of persistent sodium currents (see Zhong et al. 2007). The complete block by TTX demonstrates the dependence of the onset of Hb9 neuronal bursting activity on either extrinsic, electrotonically coupled rhythmically active neurons and/or intrinsic sodium conductances. Taken together with the data demonstrating spikelets, it is likely that Hb9 interneurons are electrotonically coupled to other rhythmically active neurons, and the bursting may be triggered by activity in this network.

### Calcium imaging reveals synchronous activity in Hb9 interneurons

If Hb9 interneurons are involved in electrotonically coupled networks, then it would be predicted that rhythmic oscillatory activity would be synchronous in nearby Hb9 interneurons. To test this hypothesis, we used two photon excitation fluorescence microscopy combined with calcium-sensitive dyes to study oscillations in calcium activity in Hb9 interneuronal clusters. In seven pairs of Hb9 interneurons in seven different slices, the addition of TEA (6 mM) and 4-AP (1 mM) evoked transient, rhythmic increases in intracellular calcium in both recorded neurons (Fig. 3Ai), whereas the majority of surrounding Fluo-3 loaded neurons (8/9 in 7 slices) did not display rhythmic calcium transients (Fig. 3B). One nearby neuron was active, but the frequency and pattern of the transients were different from Hb9 interneurons. The onset of the calcium transients in Hb9 interneuronal pairs was usually synchronous (Fig. 3A) although occasional failures were observed, whereby an “extra” transient was observed in only one neuron in a pair (Fig. 3Aii, arrow; 3 failures in 200–300 s in 2 of 6 pairs of Hb9 neurons). The mean frequency of the synchronous transients was very similar to that recorded in the electrophysiological studies (see preceding text), 0.15 ± 0.08 Hz, ranging from 0.07 to 0.30 Hz. With subsequent addition of NBQX (5 μM), AP5 (10 μM), bicuculline (10 μM), and strychnine (1 μM), the frequency decreased by 63.5% to 0.07 ± 0.03 Hz (range from 0.01 to 0.09 Hz; Fig. 3A), similar to the frequencies recorded electrophysiologically under these conditions (0.09 ± 0.02 Hz). The transients continued to occur in synchrony. These data demonstrate that the intracellular calcium activity
of Hb9 interneurons within a cluster oscillates in synchrony, even in the absence of fast synaptic transmission, consistent with their possible role in an electrotonically coupled network.

To investigate whether electrotonic coupling is involved in these synchronous, calcium transients, we applied the gap junction blocker carbenoxolone (at 100 or 200 μM, n = 4). After 60–90 min, the frequency of synchronous transients in two pairs of Hb9 interneurons was reduced (0.04 ± 0.02 compared with 0.1 ± 0.003 Hz). By 100–140 min of carbenoxolone application, all the transients in a further two pairs of Hb9 interneurons had ceased (Fig. 3Aiii). Although it might be expected that the bursting activity in individual neurons would continue asynchronously, complete cessation of bursting following carbenoxolone application has been previously demonstrated in other preparations (Placantonakis et al. 2006). Whether this reflects the role of electrotonic coupling in producing the bursting activity (Manor et al. 1997) or the actions of carbenoxolone unrelated to gap junction blockade (e.g., Rouach et al. 2003; Vessey et al. 2004) is not known. Nevertheless, carbenoxolone is commonly used as a gap junction blocker (Rozental et al. 2001), and the time course of the block of oscillations by carbenoxolone in our study is most in keeping with its effects on blocking gap junctions (e.g., see Ballantyne et al. 2004). These data provide evidence that electrotonic coupling is required for the synchronous oscillations in calcium activity in Hb9 interneurons in these conditions.

**Paired whole cell patch-clamp experiments reveal heterogeneous electrotonic coupling**

To investigate whether Hb9 interneurons are directly coupled to each other, whole cell patch-clamp recordings were obtained from ipsilateral pairs of Hb9 interneurons in the spinal cord slice. No synaptic potentials were seen in the second cell when action potentials were initiated in the first cell, indicating that between pairs in the slice, there was no evidence of monosynaptic chemically mediated synaptic transmission. To examine electrotonic coupling, current was injected into each neuron. In none of the 44 pairs did current injection in one neuron produce a voltage deflection in the other neuron. To increase the probability of detecting electrotonic coupling should it be present, we tested direct coupling in the presence of high external potassium (6–8 mM, n = 5 pairs, Fig. 4). No evidence of DC transfer was observed under these conditions, either when positive current was injected to evoke action potential firing in one cell (Fig. 4A) or negative current to hyperpolarize either cell (Fig. 4B). Similarly, no evidence of current transfer was observed in the presence of TEA (20 mM, n = 3 pairs). These data established that the Hb9 interneurons are not directly electrotonically coupled to each other in the transverse slice, and together with the preceding imaging data, raised the possibility that the Hb9 interneurons are in fact electrotonically coupled to rhythmically active non-Hb9 interneurons.

Although not unusual (Connors and Long 2004; Logan et al. 1996), dye coupling was never observed when recording from Hb9 interneurons (either singly or in pairs). Therefore the identity of the cells to which they are coupled could not be readily ascertained. However, given the recent demonstration of electrotonic coupling between GFP-positive neurons in Hb9:GFP mice (Hinckley and Ziskind-Conhaim 2006), we had readily ascertained. However, given the recent demonstration of electrotonic coupling between GFP-positive neurons in Hb9:GFP mice (Hinckley and Ziskind-Conhaim 2006), we proceeded to study whether Hb9 interneurons are electrotonically coupled to GFP-positive, non-Hb9 interneurons. We had termed such a population of neurons in this region in this strain of mouse as “type 2” neurons; these neurons can be recognized by their larger size, morphology, and electrophysiological properties (Ih-like potentials) (Wilson et al. 2005). We simultaneously recorded from nine pairs of Hb9 and type 2 neurons and found electrotonic coupling in one of the nine pairs
In that pair, action potential firing, induced by a depolarizing step (Fig. 5A) or repetitive depolarizing current pulses (Fig. 5Aii) in the Hb9 interneuron induced spikelets in the postsynaptic type 2 interneuron. In addition, injection of long (~100 ms) hyperpolarizing current pulses into the Hb9 interneuron evoked voltage deflections also in the type 2 interneuron (Fig. 5, B and C). Current transfer was reciprocal in that current injected into the type 2 interneuron resulted in
Synchronous bursting of mouse HB9 interneurons

Electrotonic coupling facilitates synchrony of burst initiation in HB9 interneurons

We next hypothesized that HB9 interneurons are synchronously active during locomotion. However, such synchrony could as readily be due to common chemical synaptic inputs as to electrotonic coupling. To investigate the possibility of common synaptic inputs, paired whole cell patch-clamp recordings were obtained from HB9 interneurons in the presence of antagonists to excitatory or inhibitory synaptic receptors.

The occurrence of spontaneous EPSPs in pairs of HB9 interneurons was analyzed in the presence of TEA, bicuculline, and strychnine (Fig. 6A, n = 4 pairs). Under these conditions, the synchrony percentage of the EPSPs was between 30 and 50%. Further, the sharp peak at 0 ms in the cross-correlogram (Fig. 6Ai), indicated that HB9 interneurons receive common excitatory input. The occurrence of IPSPs in pairs of HB9 interneurons was investigated in the presence of TEA, and the glutamatergic receptor antagonists NBQX and AP5. Under these conditions, 20–30% of spontaneous IPSPs were synchronized (Fig. 6B, n = 3 pairs), and the cross-correlogram exhibited a sharp peak at 0 ms (Fig. 6Bi), indicating that HB9 interneurons also receive common inhibitory input. Although these percentages are likely underestimated due to the limited networks contained in the slice preparation, the fact that these are not 100% synchronous indicates the possibility of asymmetric synaptic inputs that may in turn indicate that these neurons with very similar anatomical and physiological properties do not necessarily function homogeneously.

Thus far, we have provided evidence that HB9 interneurons receive some common chemical synaptic inputs, that they fire synchronously in the presence of synaptic blockers (see Fig. 3), and that they are coupled to non-HB9 interneurons. It is possible that they also receive common electrotonic input. To isolate electrotonic transmission, bicuculline, strychnine, NBQX, and AP5 were added (n = 6 pairs). In one pair, spikelets were revealed with a 30% correlation of simultaneous spikelets between the two cells. In the remaining five pairs, no spikelets were observed until the external potassium concentration was increased to 6–8 mM, following which the synchrony percentage of spikelets ranged from 10 to 30%, and there was a sharp peak in the cross-correlogram at 0 ms (Fig. 6C). The occurrence of simultaneous spikelets in the two neurons is indicative of electrotonic transmission involving a spiking neuron. The lack of current transfer between any of the 44 recorded pairs of HB9 interneurons indicates that the source of these spikelets is unlikely to be another ipsilateral HB9 interneuron and would therefore be from a third neuron. The lack of complete correlation indicates that there is more than one electrotonically connected (non-HB9) neuron, and that some of these neurons may not be connected to both HB9 interneurons recorded in the slice.

FIG. 6. Pairs of HB9 interneurons exhibit synchronous synaptic and gap junctional activity. Ai: in the presence of TEA (20 mM), bicuculline (10 μM), and strychnine (1 μM), many excitatory PSPs (EPSPs) recorded in the presence of TEA are synchronous. Bi: in a different pair, synchronous IPSPs were recorded in the presence of TEA (20 mM), NBQX (5 μM), and d-AP5 (10 μM). Bi: cross-correlogram of the data for the cells shown in Bi demonstrated that ~30% of IPSPs in this pair are synchronous. Note that in these experiments the internal solution contained 20 mM Cl⁻ so the IPSPs are reversed and the amplitude enhanced. Ci: in a 3rd pair, spikelets were isolated in the presence of high potassium (6–8 mM), bicuculline (10 μM), strychnine (1 μM), NBQX (5 μM), and d-AP5 (10 μM) and found to be synchronous. ii: cross-correlogram of the data for the cells shown in Ci demonstrated that ~25% of the spikelets were synchronous. Note that under these conditions (no TEA or 4-AP), the spikelet showed a pronounced hyperpolarizing phase, but in the presence of TEA and 4-AP, the depolarizing phase was dominant (e.g., Fig. 2).
To shed some light on the role of electrotonic coupling in the production of the rhythm, synchronization of action potentials and bursts was examined in the presence of chemical synaptic blockers (n = 4 pairs). In the presence of high external potassium conditions (6–8 mM), both cells in each of three pairs were close to threshold for action potential generation. Note failures indicated by arrowheads. In general, this occurred when the spikelets in one cell failed to bring it to threshold for action potential generation. Failures occurred with either action potentials or spikelets in the second cell (Fig. 7A). Addition of TEA (20 mM) produced bursts in three of four pairs, in which periods with a high degree of synchrony (i.e., bursts starting within <5 ms of each other, Fig. 7Bii) and rhythmicity (CV: 18.9) lasting between 3 and 12 min were observed (Fig. 7Bi), followed by periods where the bursting became less synchronous (i.e. more “failures,” Fig. 7Biii, arrowheads, and average delay between burst onsets was 21.0 ± 21.1 ms, Fig. 7Biv) and less rhythmic (CV: 42, Fig. 7Bi; iii and iv). No evidence of current transfer had been found between these pairs. Further, although the onsets of the bursts were synchronous (Fig. 7Bi), the different number of spikes in the two cells resulted in different burst durations (2.5 ± 0.8 vs. 3.4 ± 0.6 s, P < 0.05, e.g., Fig. 7Biii). This indicates that the bursts are likely initiated by activity in electrotonically coupled neuron(s) and that the spiking activity in the Hb9 interneurons depends on the intrinsic properties of these neurons themselves. The transition from the stable synchronous rhythm to the irregular bursting (e.g., transition from Fig. 7B, i–iii) was associated with both failures and spikelets failing to reach threshold, suggesting that a minimum level of excitation in the coupled network is required for maintenance of this rhythm.

**Calcium imaging in the in vitro spinal cord demonstrates firing synchrony between Hb9 interneurons and ventral root bursts**

Next we investigated whether the activity of Hb9 interneurons is related to the output of the rhythm-generating networks in the spinal cord. Because Hb9 interneurons receive common chemical synaptic inputs, it was necessary to turn to a preparation in which one can evoke rhythmic motor output in the absence of glutamatergic, GABAergic, and glycinegic transmission. Such activity can be evoked in the presence of synaptic blockers by raising the overall excitability of spinal neurons (Taccola and Nistri 2004, 2006). In isolated spinal cords, Hb9 interneurons were loaded with Fluo3-AM, and bilateral upper lumbar ventral root output was recorded. Initially, low concentrations of TEA (200 μM) and AP5 (500 μM) were added to induce bursts of activity in the ventral roots. Subsequent addition of NBQX (5 μM) and AP5 (10 μM) blocked all ventral root output. On increasing TEA and 4-AP concentrations to 6 and 1 mM respectively, rhythmic output returned in the roots. After addition of bicuculline (10 μM) and strychnine (1 μM), the amplitude of the bursts increased, and became bilaterally synchronous (n = 10 spinal cords). In addition, the bursting became more regular in frequency, and low-amplitude spontaneous activity between the bursts ceased (e.g., Figure 8A; see also C). Application of carbenoxolone (100 μM, n = 3) eliminated bursting activity in the ventral roots after 60 min, consistent with a role of electronic coupling in burst production (data not shown).

Prior to carbenoxolone application, GFP positive Hb9 interneurons exhibited rhythmic calcium transients that were similar to those observed in the slice (see preceding text) and that were synchronous with ventral root output (Fig. 8, A, D, and E; n = 8 Hb9 neurons in 4 spinal cords). (Note that some nearby GFP-negative interneurons in this preparation were also synchronously active with Hb9 interneurons. Whether these neurons are also connected to Hb9 interneurons remains to be determined.) When the transients in more than one Hb9 neuron were recorded simultaneously, they showed a high degree of synchrony with each other (n = 3 pairs; Fig. 8F, mean ± SD: 3.4 ± 4.7°C). In one of the three pairs, this synchrony was not absolute. That is, throughout the bursting activity (500 s), the
were calculated from 27 cycles (G) or from 17 cycles (D). These values indicated. The length of the vector and the “r” value indicates the concentration of phase values around the mean (Kriellaars et al. 1994). The mean phase value is represented by the direction of the vector, as 0° represents the activity is in phase (synchronous) while 180° represents anti-phase activity. The bar in A indicates the expanded region in B. B: on a faster time base, the onset of the calcium transients in Hb9 interneurons (lower gray panel) occurs in synchrony with the onset of the integrated ventral root output (upper black panel). Arrows denote large-amplitude transients in Hb9 interneuron 1 that are synchronous with both Hb9 interneuron 2 and with the ventral root bursts. Arrowheads denote small amplitude “extra” transients that are not synchronized with either ventral root bursting or Hb9 interneuron 2. C–F: polar plots of the onsets of the 2 waves of activity for 28 consecutive cycles reveal the degree of synchrony between the pairs of waveforms indicated. Clustering around 0° indicates that the activity is in phase (synchronous) while 180° represents anti-phase activity. The mean phase value is represented by the direction of the vector, as indicated. The length of the vector and the “r” value indicates the concentration of phase values around the mean (Kriellaars et al. 1994). These values were calculated from 27 cycles (C–F) or from 17 cycles (G). C: activity in both ventral roots is highly synchronized as is the relationship between activity in Hb9 interneuron 2 and ventral root activity (D). D: large-amplitude transients in Hb9 interneuron 1 are in phase with ventral root output. F: Hb9 interneuron 1 activity is in phase with Hb9 interneuron 2 activity. G: this polar plot demonstrates that the small amplitude transients in Hb9 interneuron 2 are scattered and out of phase with ventral root output.

The largest amplitude calcium transients recorded in one of the two Hb9 interneurons were synchronous with the ventral root bursts (Fig. 8E) and with the calcium transients in the second Hb9 interneuron (Fig. 8, B, arrows, and F). In addition to these large calcium transients, transients of smaller amplitude were seen that were not synchronous with either the ventral root bursts or the calcium transients of the second Hb9 interneuron (Fig. 8, B, arrowheads, and G). These extra calcium transients are similar to the failures seen in the imaging and the dual whole cell patch-clamp experiments in slice and indicate that these interneurons are not directly electrotonically coupled.

These data show that Hb9 interneurons undergo synchronous membrane potential oscillations that occur in phase with rhythmic motor activity. Taken together with the electrophysiological data, it is clear that Hb9 interneurons are involved in heterogeneous electrotonic coupling, which may provide a wider dynamic range in rhythm-generating networks (Manor et al. 1997; Soto-Trevino et al. 2005) (see DISCUSSION).

DISCUSSION

In this study, we have demonstrated that in response to TEA and 4-AP, Hb9 interneurons are rhythmically active in synchrony and that this activity depends on electrotonic coupling. We demonstrate that this electrotonic coupling is between heterogeneous neurons, i.e., between Hb9 and non-Hb9 interneurons, rather than between Hb9 interneurons in the late postnatal mouse. Further, in the in vitro spinal cord preparation, synchronous activity of Hb9 interneurons is in phase with rhythmic ventral root output. These data add to our previous electrophysiological characterization of Hb9 interneurons in motor functionally mature mice, where we demonstrated that their electrophysiological properties enable conditional oscillatory activity in response to neurotransmitters that induce locomotor activity (Wilson et al. 2005). Evidence that Hb9 interneurons are involved in locomotor output includes their expression of the activity-dependent protein, Fos, following locomotion (Wilson et al. 2005). In addition, some GFP-positive neurons in this region in early postnatal Hb9:GFP mice are synchronously active with ipsilateral ventral root output in the hemisectioned spinal cord in response to NMDA, 5-HT, and DA (Hinckley et al. 2005). Taken together, these data indicate that Hb9 interneurons are candidates for locomotor rhythmogenesis.

A recent publication demonstrated electrotonic coupling between medial lamina VIII GFP-positive interneurons in hemisectioned spinal cords of Hb9:GFP mice (Hinckley and Ziskind-Conhaim 2006). These investigators interpreted this as a demonstration of electrotonic coupling between Hb9 interneurons; however, our data do not support this conclusion. Although it is possible that we do not see electrotonic coupling in the slice because the gap junctions are either truncated dendrites or are closed in our recording conditions, we offer other more plausible explanations for the differences between their findings and the results presented here. Much of the data in the Hinckley and Ziskind-Conhaim (2006) paper was derived from early postnatal animals. At this developmental stage, there are large clusters of GFP-positive neurons in medial lamina VIII, many of which are Hb9 negative (Fig. 1, A and B) (cf. Figure 1 in Hinckley and Ziskind-Conhaim 2006). In addition, these investigators recorded from neurons that are larger, “spindle-shaped,” and ventral to the location of the Hb9 interneurons seen later in development (see Fig. 8 in Hinckley et al. 2005). Although their recordings may be from neurons that are Hb9-positive early in development, Hb9-positive neurons in that location with the morphological characteristics reported by Hinckley and Ziskind-Conhaim (2006) are not seen at functionally mature stages. It is therefore probable that at both developmental stages studied, Hinckley and Ziskind-Conhaim (2006) were recording from neurons that are not Hb9 positive. Therefore in our studies, we used strict criteria to ensure we were recording from a well-defined, Hb9-positive population.
that could be identified throughout postnatal development (see METHODS).

Interestingly, the “type 2,” GFP-positive, Hb9 negative neurons that we described previously are similar in morphology to those shown by Hinckley et al. (2005). In our studies (this paper and Wilson et al. 2005), we demonstrated that type 2 neurons are electrotonically coupled, both to each other and to Hb9 interneurons, raising the possibility that Hinckley and Ziskind-Conhaim (2006) found from type 2 interneurons. Although in the slice we only found that 1/9 pairs of Hb9-type 2 neurons were coupled (and 1/5 pairs of type 2-type 2 neurons), these numbers may be underestimates due to the truncation of dendrites during the slicing procedure. That is, we demonstrate that this coupling is present even in the slice, while recognizing that it may be more prevalent in the more intact, hemisected spinal cord in which Hinckley and Ziskind-Conhaim (2006) found >80% electronic coupling between GFP+ neurons. Thus the data presented here demonstrate heterogeneous electrotonic coupling between Hb9 and non-Hb9 interneurons.

Bursting: network and intrinsic properties

We have presented evidence that the synchrony of the onset of bursting is likely promoted by electrotonic coupling. However, the burst durations are not necessarily equivalent between Hb9 neurons and are therefore likely determined in large measure by the intrinsic properties of the neurons. We have previously shown that Hb9 interneurons have PIR mediated by a nickel-sensitive (CaV3) current. This current would be expected to contribute to the fast rise times of the bursts. Further, that the bursts are present after blockade of sodium channels by TTX (Wilson et al. 2005) and that they can be evoked by addition of barium after TEA, 4-AP, synaptic blockers, and TTX (supplemental Fig. 1) leads to the suggestion that CaV1 or CaV2 channels are involved in the maintenance of burst duration.

Role of electrotonic coupling in rhythm generating networks

Electrotonic coupling has been shown to facilitate synchronous spiking activity across pairs and populations of different types of neurons (see Connors and Long 2004 for review). The firing patterns and degree of synchrony of coupled cells depend on the coupling conductance as demonstrated by modeling studies of the crustacean stomatogastric ganglion (Sharp et al. 1992) and the mammalian inferior olive (Ozden et al. 2004). These studies show that neurons may fire independently in a weakly coupled system. As coupling strength increases, neurons may become phase-locked (i.e., in or out of phase depending on coupling parameters and intrinsic properties of the neurons). Interestingly, at high coupling conductances, oscillations completely cease (Ozden et al. 2004). In addition to synchronous firing, electrotonic coupling can also promote and/or stabilize bursting activity (Getting and Willows 1974; Skinner et al. 1999) and regulate the interburst interval (Sharp et al. 1992). Furthermore although individual neurons need not be endogenous pacemakers to elicit a synchronous rhythm in a coupled network (Manor et al. 1997; Sherman and Rinzel 1992; Smolen et al. 1993), intrinsic pacemaking properties of individual neurons within a coupled network can facilitate bursting activity (Sherman and Rinzel 1992). The combination of conditional bursting properties of Hb9 interneurons (Wilson et al. 2005) and heterogeneous electrotonic coupling (Hb9 to non-Hb9) may be necessary for stable, synchronized rhythmic output. Further, coupling between heterogeneous neurons may increase the dynamic range of the rhythm produced by a network (Soto-Trevino et al. 2005) and the repertoire of sub-threshold activity (Manor et al. 1997), similar to that proposed for bursting in heterogeneous populations of pancreatic β cells (Smolen et al. 1993). Flexibility in the dynamic range would be an important property for locomotor rhythmic networks.

Electrotonic coupling can be precisely and temporally modulated by a variety of neurotransmitters and neuromodulators (see Hatton 1998). Dopamine, acetylcholine, and second messengers, for example, can either increase or decrease junctional conductance depending on receptor subtype (DeVries and Schwartz 1989; Hatton 1998; Velazquez et al. 1997). Further, both electrical activity in the form of tetanic stimulation and the activation of metabotropic glutamate receptors (mGluR) can negatively regulate coupling strength (Landisman and Connors 2005). Interestingly, endogenous activation of mGluRs in the lamprey spinal cord regulates locomotor burst frequency (Krieger et al. 1998), and dopamine is required to elicit fictive rhythmic locomotor activity in the isolated mouse spinal cord (Jiang et al. 1999). The rhythm evoked by administration of potassium channel blockers has also been reported to be blocked by antagonists to mGluRs (Taccola et al. 2004). The mechanisms by which mGluR or dopamine activation regulate locomotion have not yet been determined, but it is conceivable that such activation (or activation of other modulatory receptors) may regulate gap junctions between rhythmic interneurons.

**Hb9 interneurons and locomotion**

Hb9 interneurons in the adult mouse have been shown to be active during overground locomotion based on their expression of Fos after a locomotor task (Wilson et al. 2005). Based on their anatomical and electrophysiological properties, we have proposed that Hb9 interneurons may be involved in the generation of locomotor rhythm (Brownstone and Wilson 2007; Wilson et al. 2005). We demonstrate here that Hb9 interneurons burst in synchrony and are electrotonically coupled to non-Hb9 interneurons. These properties of Hb9 interneurons make them candidates for involvement in the generation of locomotor rhythm.

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**References**


Synchronous bursting of mouse HB9 interneurons


