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

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Submitted 26 March 2007; accepted in final form 16 August 2007


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 intermedialateral 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-
ferred to other non-Hb9 interneurons. This heterogeneous electrophoretic 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 tribromoethanol (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 upper lumbar ventral roots (Jiang et al. 1999). Artificial cerebrospinal fluid (ACSF) was superfused during experimentation, and for the single-cell patch-clamp experiments contained (in mM) 127 NaCl, 1.9 KCl, 1.2KH₂PO₄, 2.4 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃, and 10 d-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 d-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% biocytin.

Whole cell patch-clamp recordings were obtained using differential interference contrast optics under infrared illumination with a MultiClamp 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, carbocain, bicuculline, strychnine (Sigma-Aldrich Canada, Oakville, Ontario, Canada), t-APV, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) (Tocris Bioscience) and tetrodotoxin (TTX, Alomone Labs, Jerusalem, Israel).

Immunohistochemistry

For immunohistochemical experiments (see Wilson et al. 2005), Hb9:eGFP mice were crossed with Hb9;βgal⁺/⁺ 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 overnight 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 then 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 Argon (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 can 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 weight bear and walk with their body weight supported (Jiang et al. 1999). At this age, 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 slow postinhibitory rebound not associated with an apparent I₈-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 medial 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
spinal cord in the 1st postnatal week (mice in the 2nd postnatal week (fig. 1A–G)). Transverse sections show lamina VIII in the Hb9:GFP population of Hb9 interneurons, tightly clustered pairs can be seen (arrowhead). Within the middle and right neurons that are Gal positive (red nuclear labeling, middle) are Hb9 positive (yellow in overlay, right). A: cluster of GFP-positive interneurons (left) contains both Hb9-positive interneurons (middle) and Hb9-negative interneurons (overlay, note GFP only interneurons, arrow). B: Hb9-positive interneurons (yellow in overlay, right) have an assortment of somatic shapes and morphology at early postnatal ages. C and D: pairs of tightly clustered Hb9 interneurons can be identified both in the 1st (C, arrowhead) and 2nd (D, arrowhead) postnatal week. E and F: in longitudinal sections, columns of Hb9 interneurons can be identified by their morphology and location abutting the ventral commissure. Within the population of Hb9 interneurons, tightly clustered pairs can be seen (arrowhead). Scale bars: 20 μm for A–D and F; 100 μm for E.

FIG. 1. Identification of Hb9 interneurons. Confocal projection images of transverse sections show lamina VIII in the Hb9:GFP × Hb9CreERT2/− mouse spinal cord in the 1st postnatal week (A–C; P2-4) and motor functionally mature mice in the 2nd postnatal week (D; P8). The GFP positive (green cytoplasmic labeling, left) neurons that are βGal positive (red nuclear labeling, middle) are Hb9 positive (yellow in overlay, right). A: cluster of GFP-positive interneurons (left) contains both Hb9-positive interneurons (middle) and Hb9-negative interneurons (overlay, note GFP only interneurons, arrow). B: Hb9-positive interneurons (yellow in overlay, right) have an assortment of somatic shapes and morphology at early postnatal ages. C and D: pairs of tightly clustered Hb9 interneurons can be identified both in the 1st (C, arrowhead) and 2nd (D, arrowhead) postnatal week. E and F: in longitudinal sections, columns of Hb9 interneurons can be identified by their morphology and location abutting the ventral commissure. Within the population of Hb9 interneurons, tightly clustered pairs can be seen (arrowhead). Scale bars: 20 μm for A–D and F: 100 μm for E.

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 \( F = 1 - \exp(-0.1) \approx 10\% \). 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

\[ J \text{ Neurophysiol} \cdot \text{VOL. 98} \cdot \text{OCTOBER 2007} \cdot \text{www.jn.org} \]
percentage change in fluorescence divided by the baseline fluorescence (delta Fl/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.scrd.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 (Kriellaar 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 relations 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. 2Ai). 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 that 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 μM) and strychnine (1 μM, n = 9; Fig. 2Ai). The burst duration was unaffected (1.0 ± 0.6 s, P > 0.05) and burst frequency increased compared with that of 4-AP and TEA alone (0.25 ± 0.07 Hz, P < 0.05, n = 2), indicating a shortening of the interburst phase of the cycle. In addition, the bursting became more regular (CV: 30.2 from 47.8). Together,
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, biccuculline, 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. 2Ci). 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 neurons in response to TEA and 4-AP, we proceeded to analyze the spiking data for evidence of electrotonic coupling. 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. 3Ai, 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), n-AP5 (10 μM), biccuculline (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) for 60–90 min. There were 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 hyperpolarize either cell (Fig. 4Ai) or negative current to hyperpolarize one neuron (Fig. 4Bi). 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 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

**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. 4Ai) or negative current to hyperpolarize either cell (Fig. 4Bi). 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.

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**FIG. 3. Hb9 interneurons exhibit rhythmic and synchronous calcium transients.** Ai: in response to TEA (30 mM) and 4-AP (1 mM), pairs of Hb9 interneurons exhibit calcium transients that are rhythmic and synchronous. (Dashed line, onset of the transients.) Note that in 1 instance, the transient in cell 2 begins prior to that in cell 1 with the cell 1 transient beginning at the time of further increase in the fluorescence of cell 2 (arrow). Aii: after the addition of 5 μM NBQX, 10 μM n-AP5, 10 μM bicuculline, and 1 μM strychnine, synchrony is preserved and the frequency decreases. At 1 point in the 100-s recording, a single calcium transient in cell 1 is not associated with a transient in cell 2 (arrow), in contrast to the “failure” shown in Ai. Aiii: subsequent addition of carbenoxolone (100 μM) blocked the calcium transients. Bi: GFP-positive interneurons are identified using 920 nm and Fluo-3-loaded cells are imaged using 800 nm photons. Scale bar is 5 μm. Bi: Hb9 interneurons (1, 2) exhibit synchronous calcium transients, whereas neighboring fluo3-loaded, GFP-negative cells were not active.
(Fig. 5). In that pair, action potential firing, induced by a depolarizing step (Fig. 5Ai) 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...
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) in Hb9 interneurons are synchronous (—). ii: cross-correlogram of the data for the cells shown in Ai demonstrates that 50% of the events were 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). ii: 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 and fired action potentials spontaneously and synchronously (synchrony percentage ranged from 50 to 90%). Single action potentials in one cell were synchronized with either action potentials or spikelets in the second cell (Fig. 7Ai). 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. 7Bi) and rhythmicity (CV: 18.9) lasting between 3 and 12 min were observed (Fig. 7Bi), followed by periods where the bursting became both less synchronous (i.e. more “failures,” Fig. 7Bii, arrowheads, and average delay between burst onsets was 21.0 ± 21.1 ms, Fig. 7Biv) and less rhythmic (CV: 42, Fig. 7Bii, iii and iv). No evidence of current transfer had been found between these pairs. Further, although the onset 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. 7Bi). 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 glycinergic 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 4-AP (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., Fig. 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: 3.5 ± 4.7°). In one of the three pairs, this synchrony was not absolute. That is, throughout the bursting activity (500 s), the
The large-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 hemisected 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 hemisected 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 in 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 this region in early postnatal Hb9:GFP mice are scattered and out of phase with ventral root output. 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 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 hemisected 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.
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) recorded 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) calcium 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.

ACKNOWLEDGMENTS

We thank Prof. S. Redman for support and contribution to the paired whole cell patch-clamp experiments and Dr. B. Conway for useful discussions of synchrony. We are grateful to A. Alcos for technical assistance and to G. Detillieux for revisions to the analysis software as described.

GRANTS

This work was supported by The Christopher and Dana Reeve Foundation and the Sam Schmidt Paralysis Foundation as well as by the Atlantic Innovation Fund in partnership with the Atlantic Canada Opportunities Agency, the Dalhousie Medical Research Foundation, and the QEII Health Sciences Centre Foundation.

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