Low-Threshold Calcium Currents Contribute to Locomotor-Like Activity in Neonatal Mice

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Running Head: Role of low-threshold calcium current in locomotion

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ABSTRACT

In this study, we examined the contribution of a low-threshold calcium current ($I_{Ca(T)}$) to locomotor-related activity in the neonatal mouse. Specifically, the role of $I_{Ca(T)}$ was studied during chemically-induced locomotor-like activity in the isolated whole-cord and in a genetically distinct population of ventromedial spinal interneurons marked by the homeobox gene Hb9. In isolated whole spinal cords, cycle frequency was decreased in the presence of low-threshold calcium channel blockers, which suggests a role for $I_{Ca(T)}$ in the network that produces rhythmic locomotor-like activity. Additionally, we used Hb9 interneurons as a model to study the cellular responses to application of low-threshold calcium channel blockers. In transverse slice preparations from transgenic Hb9::eGFP neonatal mice, NMDA-induced membrane potential oscillations in identified Hb9 interneurons also slowed in frequency with application of nickel when fast, spike-mediated synaptic transmission was blocked with TTX. Voltage-clamp and immunolabeling experiments confirmed expression of low-threshold calcium current and channels, respectively, in Hb9 interneurons located in the ventromedial spinal cord. Taken together, these results provide support that low-threshold T-type calcium currents play an important role in network-wide rhythm generation during chemically-evoked fictive locomotor activity.

KEYWORDS

T-type calcium current, Locomotion, Rhythm Generation, Spinal Cord, Hb9 Interneuron
INTRODUCTION

In vertebrates, neural circuits called central pattern generators produce rhythmic motor output during locomotion and are located in the spinal cord (Brown 1911; Grillner et al. 1981; Lundberg 1981). Although it is understood that the ventromedial region of the spinal cord is necessary for rhythm generation (Ho and O'Donovan 1993; Kjaerulff and Kiehn 1996), the identities of the neural components and the cellular mechanisms that participate in driving rhythmic locomotor activity are not well understood. One approach to address these issues has been to examine the neural network structure underlying the generation of rhythmic activity (Grillner 1985; Delcomyn 1987; Pearson and Rossignol 1991; Fedirchuk et al. 1998; Kiehn and Kjaerulff 1998; Whelan et al. 2000; Clarac et al. 2004; McDearmid and Drapeau 2006; Gabriel et al. 2008). In addition to studying the network structure, it is evident that understanding neuronal properties such as specific conductances (and their modulation) is critical to understanding network operation (Getting 1989; Kiehn and Harris-Warrick 1992; Harris-Warrick et al. 1995; Hess and El Manira 2001; Destexhe and Sejnowski 2002; Tobin and Calabrese 2005; Wang et al. 2006; Hayes et al. 2008; Ziskind-Conhaim et al. 2008; Gabriel et al. 2009; Li et al. 2010; Nanou and El Manira 2010). Together, these studies will lead to knowledge of how neural networks are organized and coordinated to generate rhythmic activity.

Multiple lines of evidence suggest that low-threshold calcium current ($I_{Ca(T)}$) is involved in generating rhythmic activity in various motor systems (Kim et al. 2001; Wilson et al. 2005; Llinas and Steriade 2006; Molineux et al. 2006,
Wang et al. 2011) and the expression of low-threshold T-type calcium channels in specific neuronal populations can help to support the cellular mechanisms underlying rhythm generation in many types of neurons. For example, $I_{Ca(T)}$ can contribute to burst firing and generation of synchronized oscillations in thalamic relay neurons and cerebellar Purkinje cells (Kim et al. 2001; Llinas and Steriade 2006; Molineux et al. 2006) and contribute to cellular properties that underlie network rhythm generation in lamprey spinal neurons (Wang et al. 2011). Thus, it would be prudent to study the role of these conductances in spinal locomotor activity.

In addition, if these conductances play a role at the network level, it would be important to understand their role on a cellular level. It is interesting to note that one class of ventromedial excitatory interneurons, Hb9 interneurons, have robust conditional bursting properties possibly related to strong post-inhibitory rebound, a property thought to be mediated at least in part by low-threshold (T-type, $Ca_v3$) calcium channels (Wilson et al. 2005). Even though they are unlikely to be primarily responsible for producing the rhythm (Kwan et al. 2009), their rhythmicity is related to locomotion as demonstrated by bursting correlated with locomotor activity (Hinckley et al. 2005; Kwan et al. 2009). Their bursting properties, location, and rhythmicity during locomotor activity, have led to the suggestion that Hb9 interneurons may play a role in locomotor rhythm (Brownstone and Wilson 2008; Hinckley et al. 2005; Tazerart et al. 2008; Wilson et al. 2005).
In this study, we hypothesized that T-type calcium current plays an important role in supporting rhythm generation necessary for locomotor activity, and examine the potential role that these low-threshold calcium currents play in generating and/or supporting rhythmic bursting in isolated whole spinal cords (network) and in membrane potential oscillations in Hb9 interneurons (cellular). Blocking CaV3 currents slows both the locomotor rhythm and the intrinsic membrane potential oscillations in Hb9 interneurons. Based on this similarity, we conclude that $I_{\text{Ca(T)}}$ is an important current in the generation and/or maintenance of locomotor-like rhythms in the vertebrate spinal cord.
MATERIALS AND METHODS

Animals

Experiments were performed on spinal cords isolated from transgenic mice (Hb9::eGFP and/or Hb9nlz/+; provided by Thomas Jessell) from post-natal day 0 (P0) to P26. Animals between P0 and P7 were euthanized by acute decapitation while animals P8 and older were first asphyxiated with carbon dioxide until completely unconscious and then decapitated, as recommended by the AMVA Panel on Euthanasia. For the anatomical experiments, P21-P26 animals were deeply anaesthetized with a combination of ketamine and xylazine prior to transcardial perfusion. All procedures were approved by the Institutional Animal Care and Use Committees at the University of Minnesota, Cornell University and Dalhousie University and were in accordance with National Institutes of Health and/or the Canadian Council on Animal Care guidelines.

Spinal Cord Preparation

Isolated whole cord: Animals (P0-P4) were decapitated, eviscerated and the spinal cord (~T3 to conus medularis) isolated via laminectomy in ice-cold (4°C), oxygenated (95% O₂ / 5% CO₂) low calcium Ringer's solution (in mM: 128 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 0.25 CaCl₂, 1.3 MgCl₂, 3.25 MgSO₄, 25 NaHCO₃, and 22 d-glucose). The isolated spinal cord was pinned ventral side up and superfused with oxygenated normal Ringer's solution (in mM: 111 NaCl, 3.08 KCl, 25 NaHCO₃, 1.18 KH₂PO₄, 1.25 MgSO₄, 2.52 CaCl₂, and 11 d-glucose) for 1 hour at room temperature (20–22°C) before starting the experiment.
Transverse slice: The spinal cord from T9-L2 was removed from P3-P10 mice as described above. The meninges were removed and the cord was imbedded in 3.7% agarose (Invitrogen; UltraPure Agarose) in either HEPES Ringer's solution (in mM: 101 NaCl, 3.8 KCl, 18.7 MgCl₂, 1.3 MgSO₄, 1.2 KH₂PO₄, 1.0 CaCl₂, 10 HEPES and 25 d-glucose; pH to 7.4 with NaOH) or sucrose solution (in mM: 188 sucrose, 25 d-glucose, 26 NaHCO₃, 25 NaCl, 10 MgSO₄, 1.2 NaH₂PO₄ and 1.9 KCl; pH to 7.4 with NaOH). A block of agarose containing the spinal section was transferred to a vibrating microtome (Leica, VT1000S or VT1200S), and transverse sections (200-300 μm) were cut in HEPES Ringer's or sucrose solution at 4°C, transferred immediately to an incubation chamber containing pre-warmed (30°C), oxygenated (95% O₂ / 5% CO₂) normal Ringer's solution and allowed to equilibrate for at least 30 minutes before starting the experiment.

Pharmacology

The following channel blockers were used: TTX (1 µM; to block voltage-gated sodium current), TEA (30 mM; to block voltage-gated potassium current), 4-AP (4 mM, to block fast transient potassium current), CsCl₂ (2 mM, to block hyperpolarization activated inward current), NiCl₂ (10-500 µM, to block low-threshold calcium current), SNX-482 (100 nM, to block R-type calcium current) and NNC 55-0396 (10 or 100 µM, to selectively block T-type calcium current). Pharmacological agents used to evoke endogenous membrane potential oscillations in slice preparations were N-Methyl-D-aspartic acid (NMDA 3-21 µM),
serotonin creatinine sulfate complex (5HT; 3-21 µM) and dopamine hydrochloride (DA; 15-50 µM). All agents were dissolved in the normal Ringer’s solution and applied to the spinal tissue at 20-22°C and were obtained from Sigma-Aldrich, except for SNX-482 (Alomone Labs).

Electrophysiological Recordings

**Isolated Whole Cord Preparations:** Extracellular suction electrodes were used to monitor motor neuron population activity from the ventral roots of neonatal mouse spinal cords during chemical-induced activity. Locomotor-like activity was reliably evoked in the intact spinal cord by bath superfusion with oxygenated normal Ringer’s solution at 20-22°C containing a combination of NMDA (3–10 µM), 5-HT (6–12 µM), and DA (15–20 µM) at a rate of ~2mL/min (Jiang et al. 1999). Recordings were made with suction electrodes on one to two ventral roots. Typically the left and right L2 (L2 and rL2, respectively; L2 is primarily flexor-related) roots were recorded, but in some cases ipsilateral L2 and L5 (primarily extensor-related) roots were recorded.

**Slice Preparations:** To make standard whole-cell patch recordings, slices were transferred from the warm (~30°C) incubation chamber to the recording chamber and equilibrated with oxygenated (95% O2 / 5% CO2) normal Ringer’s solution at room temperature for at least 10 minutes before starting the experiment. GFP-expressing cells were identified under epifluorescent illumination and visualized for targeted recording using infrared differential interference contrast optics (BX51WI; Olympus). The following criteria were used
to validate the identity of potential Hb9::GFP spinal interneurons: 1) GFP-positive somata located in the ventromedial spinal cord just ventral to the central canal, 2) GFP-positive somata typically arranged in groups of two to three cells (Wilson et al. 2007), 3) characteristic bipolar-like somata along the dorsoventral axis of the spinal cord, 4) a single projection fiber extending from each of the soma, and 5) key electrophysiological characteristics; lack of sag-potential during hyperpolarization steps and the presence of post-inhibitory rebound with doublet-spikes (Wilson et al. 2005; Brownstone and Wilson 2008; Kwan et al. 2009).

Patch electrodes (~8-10 MOhm) were pulled on a Flaming/Brown micropipette puller (P-97, Sutter Instruments) from borosilicate glass (1.5 mm OD, 0.86 mm ID, Warner Instruments). For current-clamp recordings, the patch electrodes were filled with the following intracellular solution (in mM): 138 K gluconate, 0.0001 CaCl₂, 10 HEPES buffer, 5 Mg-ATP and 0.3 GTP-Li, adjusted to pH 7.3 with KOH. For voltage-clamp recordings of calcium currents, the patch electrodes were filled with the following intracellular solution (in mM): 100 CsCl₂, 30 TEA-Cl, 0.5 CaCl₂, 1 MgCl₂, 10 HEPES buffer, 5 Mg-ATP, 0.3 GTP-Li, 5 NaCl and 10 EGTA, adjusted to pH 7.3 with KOH.

**Whole-cell current and voltage clamp:** For current and voltage clamp recordings, whole cell voltage was monitored and controlled with a MultiClamp 700B amplifier (Molecular Devices) at a gain of 100 (Rᵢ = 5 GOhm). Data were filtered at 30 kHz and digitized at 66 kHz. The recordings were accepted for data analysis if the resting membrane potential was more negative than -45 mV.
To isolate low threshold T-type calcium currents in Hb9 interneurons for voltage-clamp recording, we blocked Na\(^+\) channels with TTX (1 µM) and K\(^+\) channels with TEA (30 mM) and 4-AP (4 mM) added to the extracellular solution, and 100 mM CsCl and 30 mM TEA replacing the K\(^+\) in the intracellular solution. Although apparently not present in Hb9 interneurons, hyperpolarization-activated inward (I\(_{h}\)) channels were blocked with 2 mM CsCl added to the extracellular solution. To achieve a satisfactory block of Na\(^+\) and K\(^+\) currents, T-type calcium currents were recorded after at least 5 min superfusion of extracellular blockers. Low voltage step protocols were designed to separate T-type from higher threshold Ca\(^{2+}\) currents. The membrane potential was clamped to -100 mV and a series of ~200 msec depolarizing pulses up to 0 mV in 10 mV increments were applied. During voltage-clamp recording, the access resistance was monitored continually and the recordings were discarded if the access resistance changed >10% during the course of the experiment. All recorded neurons were labeled with 0.1% Sulforhodamine B (Sigma) added to the patch solution, and fluorescent images were acquired with a CCD camera (C-72-CCD, Dage MTI), a frame grabber (LG3, Scion) and imaging software (ImageJ, National Institutes of Health) for morphological identification (data not shown).

**Analysis:** A program written in MATLAB (Mathworks, Natick MA) was used to analyze the data. Extracellular ventral root voltage recordings were acquired with high-pass filtering and rectified offline. Estimates of mean burst frequency were determined from a Fourier transform, such that the initial estimate of mean burst frequency was the frequency where the Fourier transform magnitude
peaked over a frequency band from 0.1 to 5 Hz. The rectified voltage recordings were smoothed with a Gaussian-weighted moving average with 99% of the weight concentrated over an interval whose width was ¼ of the reciprocal of the estimated burst frequency (“¼-width”).

Whole-cell recordings were DC-coupled and not rectified. Estimates of mean burst frequency were determined from an autocorrelation analysis, such that the mean burst frequency was the reciprocal of the lag-time between the autocorrelation zero-lag peak and the first subsequent local peak. Voltage was smoothed via low-pass Butterworth filtering with a cut-off frequency of four-times the estimated burst frequency (zero-phase forward-backward convolution with second order Butterworth low-pass).

For both extracellular and whole-cell recordings, the occurrence times of rhythmic bursts in the smoothed voltages were determined with an algorithm that searched for local peaks and troughs over ¼-width intervals while forcing adjacent peaks and troughs to be separated by at least ¼-width, and furthermore forcing peaks and troughs to alternate. With the peaks and troughs defined, the individual “burst sections” were then defined as the interval between adjacent troughs. To determine the start of individual bursts, the burst-onset was defined as the time where the smoothed waveform rose from the first trough to 10% of the way to the next peak. Similarly, burst-termination was defined as the time where the smoothed waveform fell from the peak by 90% of the vertical distance to the next trough.
To quantify intracellular voltage amplitude, we measured the deflection of membrane potential (in mV) from trough-to-peak in each individual burst. Next, to quantify “burst strengths” for individual bursts (extra- and intracellular), we integrated the area between the smoothed voltage and the straight-line connection between burst start and stop points.

For both intracellular and extracellular recordings, the analysis program was used to determine cycle period (T; time difference between successive burst peaks), cycle frequency (reciprocal of cycle period (1 / T)), burst duration (proportion of the cycle period occupied by the burst) and burst strength (defined above) for each voltage trace. The means and standard deviations for each parameter were then determined.

**Immunohistochemistry**

*Tissue Preparation.* Double transgenic Hb9::eGFP;Hb9nilz/+ mice (background: C57BL/6J) age 21-26 days were deeply anesthetized using a mixture of 18% xylazine and 30% ketamine in saline given at a dose of 0.2mL/100g of body weight. Animals were then perfused using 4% paraformaldehyde (PFA), the spinal cords removed and post-fixed in PFA for 16-24 hours, and then cryoprotected in 30% sucrose overnight at 4°C. The lower thoracic and upper lumbar segment of the prepared cord was sectioned from the whole cord and frozen in Tissue-Tek® O.C.T Compound on the microtome stage in preparation for cutting. The tissue was cut into 40 µm sections and stored suspended in PBS (pH 7.4) at 4°C.
Immunolabeling. Sections that clearly expressed the native eGFP were washed for 30 minutes using 0.01% Tween 20 (Sigma-Aldrich, Inc) in PBS, and blocked with 10% Donkey Serum (Sigma-Aldrich, Inc) in PBS. The primary antibodies (provided by Drs. Gerald Zamponi and Terry Snutch) used were sheep anti-GFP (1:1000, Novus Biologicals, Littleton, CO), monoclonal mouse anti-β-galactosidase (1:1000, Invitrogen, Burlington, ON, or Millipore, Billerica, MA) and one calcium channel antibody, CaV3.1 (1:500), CaV3.2 (1:1000) or CaV3.3 (1:1000). For each calcium channel antibody, four (4) mice were used and ten (10) sections from each cord were selected and analyzed. After appropriate incubation, all sections were washed for 3 x 10 minutes in PBS and then incubated in secondary antibodies for 3-4 hours at room temperature: Alexa 647 donkey anti-mouse, Alexa 488 donkey anti-sheep, and Alexa 555 donkey anti-rabbit. All were used at a dilution of 1:250 and were obtained from Molecular Probes. Sections were once again washed in PBS for 30 minutes and mounted under Phosphate buffer (PB). Z-stack images (1μm optical slices) were obtained using a Zeiss Axiovert LSM 510 or 510 Meta laser scanning confocal microscope.
RESULTS

Nickel slows cycle frequency during fictive locomotor-like activity

To determine if low threshold T-type calcium current was potentially involved in organizing and/or establishing the motor pattern associated with chemical-induced locomotor-like activity in the isolated intact neonatal mouse spinal cord, we activated fictive locomotion with a combination ('cocktail') of neuroactive compounds (NMDA, 3-10 µM; 5HT, 6-12 µM; DA, 15-20 µM) (Jiang et al. 1999; Hinckley et al. 2005; Juvin et al. 2005; Zhong et al. 2007). Once a regular motor pattern was established (Fig. 1A, top trace), we added 100 µM NiCl₂, a T-type calcium channel blocker, and monitored the effect on the pattern of motor output with extracellular suction electrodes on lumbar ventral roots (Fig. 1A, middle trace). Nickel (Ni²⁺) application slowed the cycle frequency in 6 of 7 preparations examined. We restricted further analysis to those six preparations that showed sensitivity to Ni²⁺. In one preparation, the pattern slowed until the rhythmic bursting disappeared. In the remaining preparations (5 of 6; 83%), the motor pattern slowed, but the bursting activity was not abolished (Fig. 1B). There was a significant difference (F₂,₁₀ = 11.62, p = 0.002) in normalized cycle frequency among control (1.0, n = 5), Ni²⁺ application (mean = 0.38 ± 0.32, n = 5) and Ni²⁺ washout (mean = 0.82 ± 0.11, n = 3) (Fig. 1C). The effect of Ni²⁺ reversed, at least partly (Fig. 1A, bottom trace); the cycle frequency of the Ni²⁺ application group was significantly slower than the Ni²⁺ washout group (t = 2.90, p = 0.016). Interestingly, the oscillation burst strength among the groups was not significantly different (Fig. 1D; F₂,₁₀ = 0.33, p = 0.73), suggesting that the Ni²⁺
dependent frequency reduction was due to prolonging the duration of the interburst intervals. These data demonstrate that the locomotor cycle frequency but not burst strength is sensitive to Ni++, likely through its actions on calcium currents.

**Nickel concentration-response on fictive locomotor-like activity**

To determine the dose-response relation for Ni++ on chemically-induced fictive locomotor-like activity in the isolated intact spinal cord, we applied varying concentrations of Ni++ (50, 100, 200 and 500 µM) and monitored the cycle frequency. All Ni++ concentrations decreased the cycle frequency (n = 6; Fig. 2A & B). The IC50 for Ni++ was 110 µM. In some experiments (2 of 6; 33.3%), high concentrations of Ni++ (200 to 500 µM) produced an unorganized, transient motor pattern where the normal coordination state, defined by continuous bouts of low frequency bursts (~0.1 to 0.5 Hz), was replaced by bilaterally synchronous bursting in the left and right flexor-related roots (L2) (Fig. 2C).

**The effects of Ni++ are not mediated by R-type calcium channels or NMDA receptors**

Since Ni++ blocks both T- and R-type calcium channels (Kang et al. 2006; Kang et al. 2007; Gavazzo et al. 2009; Tsien et al. 1988; Todorovic and Lingle 1998; Huguenard 1996; Schneider et al. 1994; Zamponi et al. 1996), we asked whether the decrease in cycle frequency during chemically-induced locomotor-like activity was due to block of R-type calcium channels. To test this, we induced
fictive locomotor-like activity in the isolated whole cord and then applied the R-
type calcium channel blocker SNX-482 (100 nM) to the bath (Fig. 3A & B). The
cycle frequency was not significantly affected by SNX-482 (Control, mean = 0.24
± 0.04 Hz; SNX-482, mean = 0.23 ± 0.02 Hz; n = 3; t = 0.39, p = 0.72; Fig. 3C).
In addition, the burst strength was not significantly different between control
(mean = 64.4 ± 47.4 mV*sec, n = 3) and SNX-482 application (mean = 71.0 ± 55.2 mV*sec, n = 3; t = -0.16, p = 0.88; Fig. 3D). Thus, the Ni++ effect on
locomotion does not appear to be mediated by blockade of R-type calcium
channels.
Further, since Ni++ has been shown to block some classes of NMDA
receptors (Gavazzo et al. 2009), we further tested whether the block of
locomotion was due to T-channel block by using a selective T-type calcium
channel blocker, NNC 55-0396 (100 µM) (Huang et al. 2004; Li et al. 2005;
Alvina et al. 2009). In all preparations (3 of 3), application of NNC 55-0396
abolished the chemically-induced activity within 15 min (Fig. 4A & B) and did not
reverse, even with washout times up to 60 min (data not shown; see Huang et al.
2004). In contrast to Ni++ application, where a gradual decrease in cycle
frequency was observed over time (Fig. 1B), the effect of NNC 55-0396 on cycle
frequency occurred relatively abruptly (Fig. 4B). Because of the abrupt cessation
of activity, we cannot determine whether this occurred by silencing the output
(motoneurons) or the locomotor network.
Nickel effects on chemically-induced membrane potential oscillations in Hb9 interneurons

Given that Ni^{++} slows the rhythm of locomotion, it must be acting on pre-motor interneurons involved in rhythm-generation rather than solely on the output neurons (motoneurons), which are not involved in generating the rhythm. Since it has been suggested that Hb9 interneurons may play a role in locomotor rhythm generation (Brownstone and Wilson 2008; Hinckley et al. 2005; Tazerart et al. 2008; Wilson et al. 2005), we further studied the role of Ca_{v3} currents on Hb9 interneuron membrane potential oscillations. Recent work showed that our ‘cocktail’ of neuroactive compounds (NMDA, 5HT and DA) was sufficient to initiate membrane potential oscillations in Hb9 spinal interneurons when spike-mediated synaptic transmission was abolished with TTX (Wilson et al. 2005; Gordon and Whelan 2006; Ziskind-Conhaim et al. 2008). In addition, Wilson et al. (2005) raised the possibility that low-threshold T-type calcium current may be a potential contributing factor to rhythmic activity in these cells.

To determine what role T-type calcium current plays in generating membrane potential oscillations in the chemically-induced paradigm we first asked whether, in the transverse slice preparation, membrane potential oscillations in Hb9 interneurons isolated from synaptic inputs by the addition of TTX were affected by the application of Ni^{++}. In most experiments (9 of 10; 90%), Ni^{++} decreased the cycle frequency of the chemically-induced oscillations, but the rhythmic membrane potential oscillations were not completely eliminated (Fig. 5A-C). A significant reduction ($F_{2,29} = 31.9, p < 0.001$) in cycle frequency between
control (normalized to 1.0) and Ni\(^{++}\) application (mean = 0.49 ± 0.18) (Fig. 5C) was found. The reduction in cycle frequency partially reversed with Ni\(^{++}\) washout (mean = 0.67 ± 0.23) (Fig. 5C). The voltage amplitude of the chemically-induced membrane potential oscillations was also reduced by Ni\(^{++}\) application in most experiments (8 of 10; 80%) (Fig. 5A & D). A significant reduction (F\(_{2,29}\) = 6.6, p = 0.007) in voltage amplitude was seen between control (normalized to 1.0) and Ni\(^{++}\) application groups (mean = 0.66 ± 0.26) (Fig. 5D). The reduction in voltage amplitude partially reversed with Ni\(^{++}\) washout (mean = 0.78 ± 0.25) (Fig. 5A & D).

These data indicate that T-type currents are involved in chemically-induced voltage oscillations in most Hb9 interneurons. Further, the reduction of cycle frequency in chemically-induced membrane potential oscillations in Hb9 interneurons (0.49 ± 0.18) is similar to the reduction of frequency of locomotor activity following Ni\(^{++}\) application to the isolated spinal cord (0.38 ± 0.32, compare Figs. 1A-B & 5A-B; t\(_{13}\) = 0.87, p = 0.40).

**T-type calcium currents and channels are present in Hb9 interneurons**

Since chemically-induced membrane potential oscillations in Hb9 interneurons were sensitive to Ni\(^{++}\), we performed voltage clamp experiments to ask whether low-threshold, T-type calcium current (I\(_{Ca(T)}\)) was present in these cells. Under control conditions in voltage clamp, with sodium and potassium currents blocked, 11 of 26 (42%) identified Hb9 interneurons expressed a low-threshold inward calcium current (Fig. 6A). Using voltage commands from a
holding potential of -90 mV, the calcium current was activated starting at -50 mV. There was a significant secondary increase at higher voltages (≥ -20 mV), probably reflecting the recruitment of high-threshold calcium currents.

We next used Ni^{++} to fairly selectively block the low-threshold component of the calcium current corresponding to the T-current. We used steps to -30 mV, as much of the inward current is derived from low-threshold calcium current at this voltage (Hille 1992). Even though T-type currents are activated at more hyperpolarized voltages than L-type calcium current when expressed in mammalian cell line (Kaku et al. 2003) and in the sinoatrial node of the guinea-pig heart (Ono and Iijima 2005), with these voltage steps we expected that other voltage-gated calcium currents would also be activated. T-type calcium current have faster activation and inactivation kinetics, thus, to minimize confounding our measurements with contributions of other subtypes of calcium current, such as high-voltage activated current, we measured the amplitude of the peak initial inward current within 20 ms of the initiation of the voltage step from -90 to -30 mV. Under control conditions, the peak inward current was -45 ± 12 pA at -30 mV (Fig. 6B). Statistical analysis (RM-ANOVA) revealed a significant difference (F_{2,6} = 39.14, p < 0.001) in peak initial inward current between control conditions (-45.1 ± 12.1 pA, n = 4), Ni^{++} application (-21.9 ± 5.1 pA, n = 4) and Ni^{++} washout (-30.5 ± 8.7 pA, n = 4) (Fig. 6B). Although we did not investigate the identity of the currents responsible for the prolonged (> 50 msec) activity (Fig. 6A) during the voltage step, it is likely due to a minimal activation of other, higher-threshold
(N- and/or L-like) calcium currents. This voltage-clamp analysis demonstrates that Ni++ does indeed block a calcium current that is activated at low voltages.

To identify which types of CaV3 channels are present in Hb9 interneurons, we next turned to immunochemistry. Previously it was demonstrated that bursting properties of deep cerebellar neurons were related to the expression of the subtypes of CaV3 channels present (Molineux et al. 2006). In Hb9::eGFP animals, GFP is expressed in some interneurons that do not express Hb9 (Wilson et al. 2005). We therefore used double transgenic Hb9::eGFP;Hb9nlZ/+ animals in which GFP is expressed throughout the cytoplasm, and β-galactosidase is expressed in the nucleus of only Hb9+ neurons (Wilson et al. 2005). Hb9 interneurons were identified as GFP+ (green), β-gal+ (blue) neurons in medial lamina VIII. Similar to strongly bursting neurons in deep cerebellar nuclei (Molineux et al. 2006), we found that all Hb9 interneurons showed strong CaV3.1 (red) immunolabeling (n=2 animals, Fig. 7A) and were negative or very lightly labeled with anti- CaV3.3 (data not shown). CaV3.2 expression was confined to the nucleus of Hb9 interneurons (n=2 animals, Fig. 7B), as has been reported in hippocampal neurons (McKay et al. 2006). The functional significance of this nuclear expression is not clear. Taken together, these studies suggest that Hb9 interneurons express functional CaV3.1 channels on their cell membranes; these channels likely contribute to the post-inhibitory rebound and bursting properties of these neurons.
DISCUSSION

In this study, we examined the role of $I_{\text{Ca}(T)}$ in the generation of locomotor-related activity in the neonatal mouse. We provide evidence that pharmacological block of low-threshold calcium currents alters the chemically-induced locomotor-like pattern of activity in isolated whole-cord preparations as well as the conditional rhythmic properties of chemically-induced membrane potential oscillations in Hb9 interneurons in transverse slice preparations. Further, both functional and immunohistochemical evidence support that $I_{\text{Ca}(T)}$ and T-type calcium channels are expressed in at least a subset of genetically identified Hb9 interneurons. These data suggest that $I_{\text{Ca}(T)}$ plays an important role in the generation of chemically-induced fictive locomotor-related activity in the isolated whole-cord and rhythmic membrane potential oscillations in a population of ventromedial spinal interneurons possibly involved in locomotor-related activity.

In many rhythm-generating networks motor activity is determined, in part, by the intrinsic properties of the constituent neurons (Perkel and Mulloney 1974; Mulloney et al. 1981; Eisen and Marder 1984; Harris-Warrick and Sparks 1995; Roberts et al. 1995; Arshavsky et al. 1997; Marder and Bucher 2001; Pena et al. 2004). In turn, intrinsic properties are determined by the repertoire of ionic currents expressed by individual neurons (Getting 1989; Calabrese and Feldman 1997; Marder and Bucher 2001; Harris-Warrick 2002).

To generate a more complete understanding of rhythm generation in general, it is important to determine the basic membrane properties that support rhythmicity in these neurons (Calabrese and Feldman 1997).
Several lines of evidence suggest that $I_{Ca(T)}$ is involved in generating rhythmic activity by modulating or transforming the intrinsic firing pattern of neurons in various motor systems (Kim et al. 2001; Wilson et al. 2005; Llinas and Steriade 2006; Molineux et al. 2006). Post-inhibitory rebound is a common feature in rhythmic networks that is critical in generating alternating motor patterns (Satterlie 1985; Roberts and Tunstall 1990; Roberts et al. 2008; Bertrand and Cazalets 1998; Fan et al. 2000; Angstadt et al. 2005; Serrano et al. 2007). Further, $I_{Ca(T)}$ directly contributes to post-inhibitory rebound (PIR) in lamprey spinal neurons (Matsushima et al. 1993; Tegnér et al. 1997; Wang et al. 2011) and is a target of both serotonergic and dopaminergic modulation (Wang et al. 2011). Some mammalian spinal neurons also have PIR, including V2a interneurons (Dougherty and Kiehn 2010; Zhong et al. 2010) and a subset of dorsal horn inhibitory interneurons (Wilson et al. 2010) The ionic mechanisms underlying PIR in these neurons has not been described. The present study focuses on the low-threshold T-type calcium current ($I_{Ca(T)}$) in Hb9 interneurons; this current has been shown to help support neuronal membrane potential oscillations or bistability in other systems (Huguenard 1996; Destexhe and Sejnowski 2002; Swensen and Bean 2003; Molineux et al. 2006; Alvina et al. 2009). Specifically, we focused on the role of $I_{Ca(T)}$ both at the network level and at the cellular level in Hb9 interneurons since these cells: 1) are rhythmically active during fictive locomotor-like activity (Hinckley et al. 2005), 2) are intrinsic oscillators and, as such, generate rhythmic membrane potential oscillations in the presence of TTX in response to a combination of NDMA, dopamine and
serotonin ('cocktail') (Jiang et al. 1999; Wilson et al. 2005) and 3) generate a robust post-inhibitory rebound that is mediated by a low-threshold, nickel-sensitive calcium conductance (Wilson et al. 2005). Recently, Ziskind-Conhaim et al. (2008) showed that Ni++ irreversibly suppressed chemically-induced membrane potential oscillations in Hb9 interneurons (both large and small amplitude) and motoneurons and eliminated chemically-induced ventral root bursting in isolated hemisected spinal cord preparations. Here we showed that the cycle frequency of chemically-induced locomotor-like activity in isolated whole-cord preparations was reversibly decreased (Fig. 1A&C) in most (5 of 6) preparations but only completely eliminated in a single preparation. It is possible that differences in the experimental paradigms employed could produce these different results. For example, the former experiments (Ziskind-Conhaim et al. 2008) were done in isolated hemisected spinal cord preparations with specific neurotransmitter antagonists applied to synaptically isolate cells, whereas our paradigm utilized isolated intact neonatal mouse spinal cords with TTX applied to isolate the cells from fast, spike-mediated synaptic transmission. Regardless of the experimental differences, Ni++ application produced a similar decrease in cycle frequency on chemically-induced membrane potential oscillations in Hb9 interneurons and locomotor-like activity. Our results were initially ambiguous, since Ni++ also blocks R-type calcium current (Newcomb et al. 1998; Wang et al. 1999), and can also block the subset of NMDA channels that contain NR2A subunits (Gavazzo et al. 2009). To support a role for the T-type calcium current, we showed that the
specific R-type blocker SNX-482 did not perturb the chemically-induced locomotor-like activity in isolated whole-cords. Further, specific block of T-type calcium channels with the selective blocker NNC 55-0396 abolished fictive locomotion, although the path to blockade of locomotion was somewhat different (compare Figs. 1B & 4B). The difference between these data and those obtained with Ni\(^{++}\) application may result from a difference in potencies of the two compounds, different perfusion into the cord, and/or different mechanisms of action of the pharmacologic agents. Generally, these results support the hypothesis that Ni\(^{++}\), at low concentrations (~100 \(\mu\)M), acts through T-type calcium channels.

Is the reduction of cycle frequency by Ni\(^{++}\) in Hb9 interneurons causally related to the reduction in cycle frequency in the isolated spinal cord? We have noted that the effects of Ni\(^{++}\) on membrane potential oscillations seen in Hb9 interneuron whole-cell recordings (Figs. 5A,C & 6B) do not wash out, but there is washout of these effects seen in isolated spinal cord ventral root recordings (Fig. 1A,C). We speculate that differential effects by Ni\(^{++}\) on various spinal cord neurons may account for the washout differences on cycle frequency. For example, Hb9 interneurons appear to be highly sensitive to Ni\(^{++}\) and thus do not fully recover, at least over a 10-15 min washout. If, however, other non-Hb9 spinal interneurons are also sensitive to Ni\(^{++}\) but to a lesser degree, then it is likely that these cells will recover more fully and potentially with a faster time course. Thus, since the effect of Ni\(^{++}\) on network activity nearly fully recovers, it is likely that non-Hb9 interneurons (less Ni\(^{++}\)-sensitive) play a more direct role in
locomotor rhythm generation than do Hb9 interneurons (more Ni\textsuperscript{++}-sensitive).

That is, although we found that T-type calcium current was involved in providing support for rhythmic activity in the locomotor CPG and that $I_{\text{Ca(T)}}$ is present in Hb9 interneurons, it does not seem that the reduction in frequency of fictive locomotor-like activity in the whole spinal cord is a direct consequence of blocking $I_{\text{Ca(T)}}$ specifically in Hb9 interneurons. This interpretation would be consistent with a recent study suggesting that Hb9 interneurons are unlikely to be the primary kernel responsible for rhythm generation because, within an individual segment of cord, they start to fire after the motor neurons, and motor neuron rhythmicity continues even when Hb9 activity ceases (Kwan et al. 2009). Thus the evidence does not support a causal relationship between Hb9 rhythmicity and locomotor rhythm generation and in fact, $I_{\text{Ca(T)}}$ may promote rhythm generation in other neurons that are part of the locomotor central pattern generator (see Kiehn 2006; Brownstone and Wilson 2008; Hägglund et al. 2010).

In summary, our results provide convergent evidence that T-type calcium currents play an important role in generating chemically-induced membrane potential oscillations in Hb9 interneurons. Furthermore, using these interneurons as models of rhythmogenic neurons, we have demonstrated that blocking these currents has a similar effect on intrinsic membrane potential oscillations as on locomotor rhythm in the isolated spinal cord.
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**FIGURE LEGENDS**

**Figure 1.** Fictive locomotor-like activity in the isolated whole-cord is eliminated by exogenous Ni**++** application. A, Paired extracellular recordings from ipsilateral lumbar ventral roots (iL2 and iL5) in an isolated whole-cord preparation with ‘cocktail’ (3-6 µM NMDA, 5-10 µM 5-HT and 15-20 µM DA) applied to produce fictive locomotor-like activity. Top Panel, ‘Cocktail’ induced motor pattern. Middle Panel, Exogenous application of NiCl2 (+ Ni**++)** slows the rhythmic bursting of the motor pattern and reduces the burst strength. Bottom Panel, Washout of NiCl2 (- Ni**++)** reverses the effects and coordinated pattern of motor activity returns. B, Time course of cycle frequency with Ni**++** application. C, Plot of normalized cycle frequency against treatment: Control, + Ni**++** (application), - Ni**++** (washout). D, Plot of normalized burst strength against treatment: Control, + Ni**++** (application), - Ni**++** (washout). Data are normalized to ‘Control’ condition prior to application of Ni**++**. Asterisks indicate significant differences.

**Figure 2.** Concentration-response of NiCl2 on fictive locomotor-like activity in the isolated whole-cord. A, Extracellular recordings of ‘cocktail’ (3-6 µM NMDA, 5-10 µM 5-HT and 15-20 µM DA) induced fictive locomotor-like activity from a single ventral root (L2) at various NiCl2 concentrations. Cycle frequency decreases and is ultimately eliminated as the concentration of NiCl2 increases. B, Concentration–response curve showing the effects of NiCl2 on cycle frequency. Cycle frequency is normalized to NMDA condition prior to application of NiCl2. The curve was fit with the equation 1/(1 + ((D/IC50)^n), where [D] is the NiCl2
concentration, IC$_{50}$ is the dose for half-inhibition, and $n$ is the Hill coefficient. IC$_{50}$ of 110 µM. Data are normalized to ‘Control’ condition prior to application of Ni$^{++}$.

C, In some preparations (2 of 6; 33%), high concentrations of NiCl$_2$ (~200 µM) produced a transient unorganized motor pattern. Note that the motor pattern was disrupted under these conditions: 1) the left and right ventral root recordings from lumbar segment 2 (iL2 and cL2) produced a synchronous activity pattern rather than the typical alternating pattern and 2) the bursting occurred at a frequency higher than normally observed.

Figure 3. Application of the selective R-type calcium channel blocker SNX-482 does not alter fictive locomotor-like activity. A, Paired extracellular recordings from ipsilateral ventral roots (iL2 and iL5) in an isolated whole-cord preparation with ‘cocktail’ (3-6 µM NMDA, 5-10 µM 5-HT and 15-20 µM DA) applied to produce fictive locomotor-like activity. Left, ‘cocktail’ induced motor pattern. Right, Exogenous application of SNX-482 (+ SNX-482) does not alter the rhythmic motor pattern. B, Time course of cycle frequency with SNX-482 application. C, Plot of normalized cycle frequency against treatment: Control and + SNX-482 (application. D, Plot of normalized burst strength against treatment: Control, + SNX-482 (application. Data are normalized to ‘Control’ condition prior to application of SNX-482. Asterisks indicate significant differences.

Figure 4. Application of the selective T-type calcium channel blocker NNC 55-0396 eliminates fictive locomotor-like activity. A, Single extracellular recording
from ventral root L2 in an isolated whole-cord preparation with ‘cocktail’ (3-6 µM NMDA, 5-10 µM 5-HT and 15-20 µM DA) applied to produce fictive locomotor-like activity. Top Trace, ‘Cocktail’ induced motor pattern. Middle Trace, Exogenous application of NNC 55-0396 (+ NNC 55-0396) slows the rhythmic bursting of the motor pattern and reduces the burst strength. Bottom Trace, Exogenous application of NNC 55-0396 ultimately (~10 min) eliminates the motor pattern; this effect is irreversible over the time course monitored (data not shown). B, Time course of cycle frequency with NNC 55-0396 application.

Figure 5. Chemically-induced membrane potential oscillations in Hb9 interneurons are sensitive to Ni++. A, Whole-cell current-clamp recordings of membrane potential oscillations in an Hb9 interneuron in the presence of a ‘cocktail’ of chemicals (21 µM NMDA, 21 µM 5-HT and 50 µM DA). Top Trace, Chemically-induced membrane potential oscillations when fast, spike-mediated synaptic events are blocked by TTX. Middle Trace, Exogenous application of Ni++ reduces cycle frequency and voltage amplitude. Bottom Trace, The effects of Ni++ reverse following washout. B, Time course of cycle frequency with Ni++ application from a representative Hb9 interneuron. C, Plots of normalized cycle frequency (raw mean at 0 min = 0.7 ± 0.2 Hz, range = 0.3 to 1.0 Hz, n = 10) against treatment condition. D, Plot of normalized voltage amplitude (raw mean at 0 min = 17.8 ± 10.4 mV, range = 5.8 to 34.0 mV, n = 10) against treatment condition. Data are normalized to NMDA condition prior to application of Ni++. Asterisks indicate significant differences.
Figure 6. Ni\textsuperscript{++} reversibly blocks T-type calcium current in Hb9 interneurons. A, Calcium currents elicited in response to voltage steps from -90 mV to -30 mV. Ni\textsuperscript{++} (200 μM) reduced the calcium current. The reduction was reversed by a washout of Ni\textsuperscript{++}. Each step is the average of 4 identical voltage steps during the given application. Arrows represent peak initial inward current within the first 20 msec of the voltage step. B, Average I-V plot of elicited currents. Points represent the amplitude of the peak initial inward current (mean +/- SD) elicited by steps from -90 mV to potential on x-axis. Black squares: Control. Gray triangles: 200 μM Ni\textsuperscript{++}. Gray squares: Washout. Note the -30 and -20 mV steps with a reversible reduction of current. Points are offset on x-axis to highlight error bars (SD).

Figure 7. Ca\textsubscript{v}3 channels are expressed in Hb9 interneurons. Immunohistochemistry in Hb9::eGFP;Hb9\textsuperscript{nlz/+} mice demonstrates the distribution of Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels in the ventral spinal cord (A\textsubscript{1}, B\textsubscript{1}). At high magnification of the region of Hb9 interneurons (indicated by arrows), Ca\textsubscript{v} channel expression (red, A\textsubscript{2}, B\textsubscript{2}) can be studied in Hb9 interneurons that can be identified by expression of eGFP in the cytoplasm (green, A\textsubscript{3} and B\textsubscript{3}) and lacZ in the nucleus (blue, A\textsubscript{4} and B\textsubscript{4}). Hb9 interneurons express Ca\textsubscript{v}3.1 channels in the cytoplasm and possibly membrane (overlay A\textsubscript{5}). On the other hand, Ca\textsubscript{v}3.2 channels are seen in the nucleus of Hb9 interneurons (overlay, B\textsubscript{5}).
Figure 1

Anderson, Abbinanti, Peck, Gilmour, Brownstone, Masino

A

+Cocktail'

iL2

iL5

+ Ni++ (100 μM; 10 min)

washout Ni++

B

Cycle Frequency (Hz)

0.0

0.2

0.4

+ Ni++ (100 μM)

Time (min)

0

2

4

6

8

10

12

14

C

Cycle Frequency (normalized)

* *

Control + Nickel - Nickel

D

Burst Strength (normalized)

Control + Nickel - Nickel
Figure 2
Anderson, Abbinanti, Peck, Gilmour, Brownstone, Masino

A

'Cocktail'

+ 50 μM Ni^{++}

+ 100 μM Ni^{++}

+ 200 μM Ni^{++}

+ 500 μM Ni^{++}

5 sec

B

Cycle Frequency (normalized)

NiCl\(_2\) (μM)

[C\(_{50}\) = 110μM]

C

'Cocktail' + 200 μM Ni^{++}

iL2

cL2

5 sec
Figure 3

Anderson, Abbinanti, Peck, Gilmour, Brownstone, Masino

A

'B Cocktail' + SNX-482 (100 nM; 15 min)

B

Cycle Frequency (Hz)

Time (min)

C

Cycle Frequency (normalized)

Control + SNX-482

D

Burst Strength (normalized)

Control + SNX-482
Figure 4

Andersen, Abbinanti, Peck, Gilmour, Brownstone, Masino

A

'Cocktail'

+ NNC 55-0396 (100 μM; 7 min)

+ NNC 55-0396 (100 μM; 15 min)

B

[Graph showing cycle frequency over time with NNC 55-0396 (100 μM)]
Figure 5
Anderson, Abbinanti, Peck, Gilmour, Brownstone, Masino

A
'Cocktail'

+ Ni++ (100 μM)

washout Ni++

B
+ Ni++ (100 μM)

C
Cycle Frequency (normalized)

0.0 0.5 1.0 1.5

Control  + Nickel  - Nickel

D
Voltage Amplitude (normalized)

0.0 0.5 1.0

Control  + Nickel  - Nickel
Figure 6
Anderson, Abbinanti, Peck, Gilmour, Brownstone, Masino

A

-30 mV
-90 mV

Control
+200 μM Ni++
Wash

B

$V_m$ (mV)

Peak Current (pA)

-70 -60 -50 -40 -30 -20 -10 0

Control
+200 μM Ni++
Wash
Figure 7
Anderson, Abbinanti, Peck, Gilmour, Brownstone, Masino