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Synaptic integration of rhythmogenic neurons in the locomotor circuitry: the case of Hb9 interneurons

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Abstract

Innovative molecular and genetic techniques have recently led to the identification of genetically defined populations of ipsilaterally projecting excitatory interneurons with probable functions in the rhythm-generating kernel of the central pattern generators (CPGs). The role of interneuronal populations in specific motor function is determined by their synaptic inputs, intrinsic properties, and target neurons. In this review we examine whether Hb9-expressing interneurons (Hb9 INs) fulfill a set of criteria that are the hallmarks of rhythm generators in the locomotor circuitry. Induced locomotor-like activity in this distinct population of ventral interneurons is in phase with bursts of motor activity, raising the possibility that they are part of the locomotor generator. To increase our understanding of the integrative function of Hb9 INs in the locomotor CPG, we investigated the cellular mechanisms underlying their rhythmic activity and examined the properties of synaptic inputs from low-threshold afferents and possible synaptic contacts with segmental motoneurons. Our findings suggest that the rhythmogenic Hb9 INs are integral components of the sensorimotor circuitry that regulate locomotor-like activity in the spinal cord.

Keywords

locomotor-like rhythms; rhythmogenic interneurons; Hb9 interneurons; rhythm-generating kernel; locomotor central pattern generator; Hb9:eGFP transgenic mouse

Introduction

In all walking vertebrates, alternating hindlimb movements on the two sides of the body are controlled by the locomotor central pattern generators (CPGs), spinal networks that can generate a coordinated pattern of motor rhythms independently of sensory inputs.^{1–4} In the isolated spinal cord, in the absence of descending inputs, the autonomous CPG can be stimulated to produce coordinated locomotor-like rhythms in the isolated spinal cord by either exposure to various neurotransmitter agonists^{5–11} or by excitation of sensory afferents.^{12–15} The CPGs for flexor- or extensor-related activity comprise ipsilateral excitatory interneurons that form the rhythm-generating kernel and commissural interneurons that coordinate the left–right alternating patterns of rhythmic activity.¹⁶

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Conflicts of interest

The authors declare no conflicts of interest.

Ipsilaterally projecting inhibitory interneurons are responsible for the alternating rhythms between flexor- and extensor-related motoneurons. Classical electrophysiological approaches combined with pharmacological manipulations have identified interneuronal populations that are part of the sensorimotor networks in the cat spinal cord.^{17–19} These studies provided important information about the complex interactions between sensory inputs and interneurons in the locomotor networks, but because of technical limitations manipulating interneurons with various functions, the organization of these interneurons in the locomotor circuitry remain largely unknown.

Experimental manipulations helped identify groups of interneurons that might play a role in rhythm-generation and pattern coordination of motor activity in the isolated rodent spinal cords. Electrophysiological and morphological studies have characterized several classes of inhibitory interneurons that have been identified as key components in rhythm-coordinating networks.^{20–23} However, up until a few years ago little was known about the identity of rhythmogenic interneurons that provide excitatory drive to ipsilateral motoneurons in quadrupeds. The development of novel molecular and genetic techniques^{24,25} facilitated the discovery of genetically defined excitatory neuronal populations that might play a fundamental role in controlling rhythmic excitation in corresponding motoneurons. These populations include interneurons that express the Hb9 transcription factor (Hb9 INs),^{26,27} the EphA4-positive neurons,²⁸ V2a interneurons,^{29,30} and V3 interneurons.³¹ Ablation or silencing the latter three classes of interneurons significantly altered the coordinated patterns of rhythmic motor activity, leading to the conclusion that they are essential components of the locomotor CPGs. These exciting findings are the first step in the identification of interneuronal populations with possible role in rhythmogenesis. To gain insight into the specific function of these interneurons in the complex architecture of the locomotor circuitry future studies will have to: (1) determine the regulatory actions of peripheral sensory projections and descending inputs on their activity patterns, (2) classify their intrasegmental synaptic interactions with other groups of interneurons with known functions, and (3) identify their target neurons.

Our review focuses on Hb9 INs, a cluster of rhythmogenic interneurons that fulfill important criteria that define a specific group of neurons as part of the rhythm-generating kernel of the locomotor CPG.³² We briefly summarize the properties that make Hb9 INs appealing candidates for ipsilateral CPGs and introduce new findings that support the concept that they are integrated in the sensorimotor circuitry that modulate rhythmic motor activity.

Properties of Hb9 INs: hallmarks of rhythm-generating neurons

The discovery of Hb9 INs is credited to the development of the Hb9:eGFP transgenic mouse line³³ in which a group of Hb9:GFP interneurons can be visually identified for repeated experimental procedures.^{26,27} Hb9 INs are clustered in medial lamina VIII of the lower thoracic and upper lumbar segments of the cord (Fig. 1), which are considered “hot” rhythmogenic areas along the rostrocaudal and ventrodorsal axes of the cord.^{34,35} The interneurons are also visible in medial lamina VIII of cervical but not sacral segments (unpublished data). Their location and morphological appearance facilitate their visual identification, but their identity is confirmed unambiguously by a set of established electrophysiological criteria.^{27,36,37}

Hb9 INs drew significant attention in recent years because they were the first class of genetically identified locomotor-related interneurons with probable role in rhythmogenesis in the isolated spinal cord of neonatal mice. They possess several properties that substantiate their putative contribution to rhythm generation in predominantly flexor-related motoneurons in upper lumbar segments L1–L3.³⁸ The main characteristics are: (1)

Neurochemically induced locomotor-like voltage oscillations are in-phase with ipsilateral motor activity in flexor-related segments (L1–L3, Fig. 1A).^{26,36,39} (2) The induced membrane oscillations persists in the absence of fast primary excitatory and inhibitory synaptic transmission (Fig. 1c).^{27,36,37,39–41} (3) Electrical coupling between them (Fig. 1B)⁴⁰ and with adjacent interneurons³⁹ synchronizes their outputs to target cells and might contribute to the synchronization of network activity. Although these observations raise the intriguing possibility that Hb9 INs are important components of the rhythm-generating circuitry, their synaptic integration in the complex locomotor networks remains unknown. Examining their intrasegmental synaptic connections is a crucial step in shedding light into their functional hierarchy in the locomotor circuitry. However, it should be emphasized that even if Hb9 INs are part of the generator that dictates the timing of motoneurons excitation, it is unlikely that they are the sole timing controller for flexor-related activity. Hindlimb movements require coordinated excitation of many groups of flexor muscles that are probably controlled by numerous synaptically connected rhythm generators.

A recent study has demonstrated that during neurochemically induced rhythmic activity, the onset of action potential firing in the majority of Hb9 INs lags the start of ipsilateral ventral root burst.⁴² Moreover, rhythms generated by stimulating the conus midualis of the cord are more variable in Hb9 INs than in corresponding motoneurons so that the phase correlation between them is not maintained. Based on these findings, it has been proposed that that Hb9 INs cannot serve as the sole rhythm generator at the upper lumbar segments. These interesting findings should be interpreted cautiously. Ventral root bursts in segments L1–L3 represent motoneuron activity in both the medial and lateral motor columns. Motoneurons in the two columns have different functions: the lateral motoneurons innervate the limb, while medial motoneurons innervate primarily axial musculature and some hip flexor muscles.⁴³ It is conceivable the two groups of motoneurons are active at a slightly different time and Hb9 INs regulate the burst onset of the slower motoneuron population. Moreover, sympathetic preganglionic also project out of segment L1 and L2 and their activity, which may be synchronized with somatic motoneurons⁴⁴ could precede the rhythms generated by Hb9 INs and somatic motoneurons. The fact that stimulation of the caudal cord produces irregular rhythms in Hb9 INs can be explained if different CPGs are activated by descending and ascending projections. It is unknown whether stimulation of the conus midualis of the cord, which co-excites commissural and ipsilateral ascending/proprio-spinal pathways⁴⁵ activates the same CPGs that are controlled by descending/proprio-spinal networks.⁴⁶ Hb9 INs might receive their primary regulatory inputs from descending pathways.

Cellular mechanisms underlying rhythm generation in Hb9 INs

Intrinsic properties that contribute to voltage membrane oscillations

Our experiments were carried out using the hemisected mouse spinal cord with a longitudinal split that severed contralaterally projecting axons, thereby eliminating the contribution of commissural networks.²⁶ Unless otherwise stated, locomotor-like rhythms were generated by rhythmogenic cocktail containing: *N*-methyl-DL-aspartic acid (NMA, 5 μ M), 5-hydroxytryptamine creatinine sulfate complex, (5-HT, 10 μ M) and dopamine (50 μ M).

Spontaneous repetitive firing is not apparent in Hb9 INs, but endogenous episodes of membrane voltage oscillations similar to those induced by the rhythmogenic cocktail can be recorded periodically (Figs. 1B and 2A). Similarly, spontaneous oscillatory activity emerge in these neurons after removing extracellular calcium.⁴¹ Bursts of spontaneous excitatory postsynaptic currents (EPSCs) (Fig. 1B₁) might initiate some of the endogenous firing episodes in the highly excitable Hb9 INs (input resistance ranging from approximately 1.0–1.5 G Ω). Typically, non-Hb9 interneurons in medial laminae VII–VIII fire at relatively

constant frequency of 3–7 Hz (Fig. 2A). The constant firing might be attributed to the large (>10 mV) action potential afterhyperpolarization and the hyperpolarization-dependent depolarization sags that characterized these interneurons (Fig. 2A and B). Hb9 INs are characterized by a relatively small afterhyperpolarization and linear current-voltage relationship without the sag. Two of their cellular properties that are likely to contribute to the episodes of rhythmic firing include: (1) strong postinhibitory rebound and (2) spike frequency adaptation (Fig. 2B).

Voltage-dependent currents that underlie induced voltage oscillations

The ability of rhythmogenic neurons to produce subthreshold voltage oscillations relies on a dynamic interplay between voltage-dependent ionic currents and signal transduction pathways independently of fast synaptic transmission.⁴⁷ Fast and slow synaptic transmission regulates the onset and termination of rhythmic activity in unidentified spinal interneurons and commissural interneurons,^{48,49} but not in Hb9 INs (Fig. 3). Blocking fast excitatory and inhibitory synaptic inputs does not alter the frequency of neurochemically induced voltage oscillations in Hb9 INs (Fig. 3A).³⁷ To elucidate the voltage-gated currents that mediate the slow membrane oscillations independently of synaptic transmission, experiments were performed in synaptically isolated hemisected spinal cords (in the presence of CNQX, picrotoxin and strychnine; antagonists of non-NMDA, GABA_A, and glycine receptors, respectively). The importance of persistent sodium current (I_{NaP}) to rhythm generation has been demonstrated in neuronal groups associated with rhythmic motor outputs controlling respiratory functions and mastication,^{50–53} but see Ref. ⁵⁴. Therefore, we examined whether it plays a role in mediating voltage oscillations in Hb9 INs. Exposure to rhythmogenic cocktail containing low concentrations of NMA (5 μ M), 5-HT (10 μ M) and dopamine (50 μ M) triggered relatively small (<10 mV) and slow (~0.1 Hz) locomotor-like voltage oscillations that were blocked by riluzole (10 μ M), a blocker of I_{NaP} (Fig. 3B) and TTX (1 μ M) that blocks both the fast- and slow-inactivating sodium channels. These data indicate that I_{NaP} is essential for generating the induced membrane oscillations in Hb9 INs. I_{NaP} plays an important role in controlling rhythmic activity in Hb9 INs following the removal of extracellular calcium.⁴¹ The current is activated at subthreshold potentials (average of –58 mV³⁷), which might contribute to the synchronization of network activity as hypothesized in the respiratory circuitry.⁵⁵ Our finding that I_{NaP} regulates locomotor-like rhythmic activity in Hb9 INs independently of fast synaptic transmission provides additional support to the hypothesis that they function as integrated components in the rhythmogenic locomotor circuitry.

Recently it became apparent that increasing the concentration of *N*-methyl-D-aspartic acid (NMDA, 20 μ M) in the rhythmogenic cocktail triggers different ionic mechanisms that generate larger (>10 mV) and faster (0.2–0.3 Hz) voltage oscillations than those produced during exposure to lower NMA concentration (5 μ M).³⁷ TTX does not alter the frequency of the faster subthreshold oscillations but it reduces their amplitude, implying that currents stronger than I_{NaP} control their frequency.^{27,36,37} NMDA receptor activation alone is sufficient to trigger voltage oscillations in motoneurons and interneurons near the central canal.^{56,57} Similarly, exposure to high concentration of NMDA (10–20 μ M) can produce rhythmic activity in Hb9 INs that are not regulated by I_{NaP} (not shown). It has been proposed that low-voltage activated (LVA) calcium current underlies the large amplitude membrane oscillations produced by rhythmogenic cocktail with high concentration of NMDA.²⁷ The conclusion is based primarily on the observation that nickel, a relatively specific blocker of LVA calcium current, suppresses the rhythmic activity. We have also documented that nickel (100 μ M) blocks both the small and large membrane oscillations in synaptically isolated Hb9 INs (Fig. 4A). However, our preliminary data show that nickel-sensitive calcium current cannot be evoked by step depolarizations from a holding potential

of -60 mV to -40 mV confirming the generally accepted assumption that LVA calcium current is partially inactivated at resting membrane potential. Small nickel-sensitive calcium currents can be generated by step depolarizations from a holding potential of -90 mV to -60 mV (not shown). The finding that LVA calcium current cannot be produced at membrane potential of -60 mV requires an alternative explanation for the action of nickel on induced locomotor-like oscillations.

It is conceivable that nickel blocks NMDA receptor-mediated currents as reported in cultured neurons.⁵⁸ To test this possibility, we examined the effect of mibefradil, another relatively specific blocker of LVA calcium current^{59,60} on voltage oscillations triggered by high NMDA concentration. Mibefradil ($10\text{ }\mu\text{M}$) as well as nickel ($100\text{ }\mu\text{M}$) blocked the small ($4\text{--}7$ mV) rebound postinhibitory potentials (RIPs) generated in the presence of TTX in response to a break from hyperpolarizing current pulse to ~ -100 mV ($n = 5$ Hb9 INs). The RIPs are thought to be mediated by LVA calcium current. In synaptically isolated Hb9 INs, the amplitude or frequency of membrane oscillations produced by NMDA ($20\text{ }\mu\text{M}$) did not significantly change during up to 40 min exposure to mibefradil ($10\text{ }\mu\text{M}$) (Fig. 4B). The mean amplitude and frequency of subthreshold oscillations were $8.0\text{ mV} \pm 2.3$ (SE, $n = 3$) and $0.35\text{ Hz} \pm 0.04\text{ Hz}$ (7.2 ± 1.7 mV), respectively before mibefradil application and 7.2 ± 1.7 mV and $0.44 \pm 0.11\text{ Hz}$ in its presence. Mibefradil had no effect on amplitude and frequency of lower frequency oscillation (0.07 Hz) induced by cocktail with low NMA concentration ($5\text{ }\mu\text{M}$, $n = 2$). Mibefradil-resistant membrane oscillations were blocked by nickel (Fig. 4B). Therefore, it is possible nickel suppresses induced locomotor-like membrane oscillations by blocking NMDA receptors rather than LVA calcium current.

It is reasonable to assume that the slow voltage oscillations that are generated by I_{NaP} (low NMA) are terminated by the slow inactivation of persistent sodium channels.^{61,62} However, little was known about the current responsible for terminating the faster oscillations triggered by high NMDA. We hypothesized that if NMDA produces a large calcium influx, it might activate calcium-dependent potassium channels that play a role in burst termination in rhythmic neurons in the lamprey.⁶³ Exposure to charybdotoxin (100 nM), a blocker of high conductance calcium-dependent potassium channels (BKCa), did not suppress membrane oscillations triggered by low-NMA rhythmogenic cocktail (Fig. 5A), but the rhythms became less regular in its presence. An average amplitude of 7.2 ± 0.6 mV (S.E., $n = 3$) and frequency of $0.24 \pm 0.04\text{ Hz}$ were recorded before charybdotoxin application, changing to 5.2 ± 1.9 mV and $0.4 \pm 0.22\text{ Hz}$ in its presence. In contrast, the blocker eliminated voltage oscillations induced by high-NMDA cocktail (Fig. 5B), indicating that BKCa channels play an important role in voltage oscillations triggered by high NMDA. The contribution of calcium-dependent potassium currents to shortening the duration of oscillatory activity might explain the higher frequency membrane oscillations generated by a cocktail with higher NMDA concentration. Apamin (100 nM), a blocker of the small conductance calcium-dependent potassium channels, did not affect the properties of high NMDA-induced voltage oscillations (not shown).

Monosynaptic inputs from low-threshold afferents modulate locomotor-like rhythms in Hb9 INs

The activity pattern of motor circuits is influenced by peripheral sensory inputs that adjust locomotor activity to external stimuli. To increase our understanding of the possible role of Hb9 INs in the locomotor circuitry, we examined whether their rhythmic activity is modulated by low-threshold afferents. In the hemisectioned spinal cord, stimulating low-threshold afferents (two time threshold) generated short-latency EPSCs in 62% of Hb9 INs (Fig. 6). These were monosynaptic EPSCs based on their stable latency during high-

frequency stimulation and their resistance to mephenesin, a barbiturate that suppresses polysynaptic responses.⁶⁴

Concomitant morphological analysis revealed that most boutons that expressed vesicular glutamate transporter-1 (VGluT-1) and parvalbumin, markers of primary afferents in neonatal rodents^{65,66} were in close apposition to dendrites of neurobiotin-filled Hb9 INs (Fig. 6, $n = 4$). Quantitative analysis showed that most of the putative synaptic contacts were on distal dendrites. The number of boutons in close proximity to tertiary dendrites was two- to threefold higher than that on primary dendrites.⁶⁴ The morphological data strongly support the electrophysiological findings that low-threshold afferents, presumably muscle afferents, provide direct sensory inputs to the majority of Hb9 INs.

If Hb9 INs are part of the locomotor networks, inputs from muscle afferents are likely to regulate their induced locomotor-like rhythms. In recent experiments we have shown that excitation of flexor-related, low-threshold afferents (L1–L3) during the flexor phase reset the rhythms in both Hb9 INs and segmental motoneurons while maintaining the phase relation between them.^{64,67} These findings provide additional support to the hypothesis that Hb9 INs are part of the sensorimotor circuitry that contribute to locomotor rhythm generation.

Putative synaptic contacts between Hb9 INs and ipsilateral motoneurons

Identifying the target neurons of a given interneuronal population is fundamental for the understanding their role in a network of known motor function. Several models have been proposed to describe the interactions between excitatory neuronal components of the CPG and motoneurons. Based on the single-level CPG, the rhythm generators directly excite functionally related motoneurons, while in more complex architectures that consist of two- and three-level CPGs, motoneurons are driven by excitatory interneurons that are part of the pattern formation networks or last order interneurons.⁶⁸ Therefore, if the rhythmogenic Hb9 INs synapse onto segmental motoneurons they might function as the generator based on the single-level model but not according to the two-level network organization.

We have previously shown that axons of neurobiotin-filled Hb9 INs project toward ipsilateral motoneuron nuclei and their bouton-like varicosities are in close apposition to GFP-expressing proximal dendrites of large neurons in lamina IX.²⁶ The neurobiotin-filled boutons express VGluT2, a marker of glutamatergic interneurons,^{26,29,31} implying that Hb9 INs form synaptic contacts onto motoneurons in the medial and/or lateral motor columns. However, in that study we could not rule out the possibility that the VGluT2-expressing boutons are in close proximity to GFP positive dendrites of excitatory interneurons rather than motoneurons. Moreover, a different study has reported that contacts between GFP positive and VGluT2-expressing boutons and motoneurons are rare in spinal cords of juvenile mice, concluding that it is unlikely that Hb9 INs have a significant number of inputs onto motoneurons.²⁷

We have recently begun a new set of experiments in which motoneurons were identified by the enzyme choline acetyltransferase (ChAT) and we investigated whether neurobiotin-filled axon terminals were in close proximity to ChAT-expressing motoneurons. Preliminary experiments have identified putative synaptic contacts between neurobiotin-filled boutons and ChAT-expressing motoneurons in the medial motor column (Fig. 7). The identity and properties of synaptic transmission between Hb9 INs and motoneurons will have to be carefully examined using paired whole-cell recordings.

Conclusions

Hb9 INs have been examined in great details by several groups and accumulated data support the concept that they are part of the rhythm-generating kernel of the locomotor CPG.

However, one of the most conclusive tests, ablation or silencing Hb9 INs to determine the effects on locomotor activity has not been performed. The Hb9 gene that distinguishes Hb9 INs from most glutamatergic interneurons is also expressed in motoneurons, therefore genetic manipulations that alter their function will disrupt motoneuron activity. Testing the effect of silencing Hb9 INs on rhythmic motor activity is important for elucidating their probable role in the locomotor circuitry, but their functional hierarchy as rhythm generator, pattern formation neurons or last order interneurons will be determined by: (1) the regulation of their activity by descending pathways, (2) their synaptic interactions with excitatory and inhibitory interneurons that are thought to be part of the rhythm-generating and rhythm-coordinating networks, and (3) the identification of their target neurons.

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Figure 1.

Locomotor-like rhythms in Hb9 INs. (A) Neurobiotin-filled Hb9 IN in medial lamina VIII, adjacent to 2–3 GFP+ putative Hb9 INs. Axon projection is visible in the proximity of motoneurons in the medial motor column. Subthreshold membrane oscillations and firing episodes in a Hb9 IN (red) are in-phase with ventral root bursts (green). (B) Spontaneous, synchronous burst of excitatory postsynaptic currents (B₁) and prolonged firing (B₂) recorded in two sets of electrically coupled Hb9 INs. (C) Synchronous, neurochemically induced subthreshold membrane oscillations in electrically coupled Hb9 INs. TEA (10 mM) and higher Ca²⁺ concentration (3 mM) were included in the extracellular solution. The similar wave form voltage oscillations were attributed to the homogeneous electrophysiological

properties of Hb9 INs. Marked traces (*dotted box*) were expanded to show their similarities (*right-hand panel*). The rhythms persisted in the presence of CNQX (10 μ M), indicating that they were independent of primary excitatory inputs mediated via non-NMDA receptors. (Modified with permission from Hinckley *et al.*, 2005 and Hinckley and Ziskind-Conhaim, 2006).^{26,40}

Figure 2.

Comparison of intrinsic properties of Hb9⁺ and adjacent Hb9⁻ negative interneurons. **(A)** Differences between action potential waveforms and spontaneous firing in Hb9⁺ and Hb9⁻ INs in hemicords of newborn mice. Hb9⁺ INs generate smaller afterhyperpolarizations than Hb9⁻ INs. Infrequent, small membrane oscillations (3–5 mV) and firing episodes develop spontaneously in Hb9⁺ INs, while firing at relatively constant frequency (5 Hz in this example) characterize adjacent Hb9⁻ INs. **(B)** Current-clamp recordings from a different Hb9⁺ IN and a neighboring Hb9⁻ IN. Resting membrane potentials were ~ -60 mV in both interneurons. Spike-frequency adaptation is apparent Hb9⁺ IN. Linear *I*-*V* relationships at potentials more negative than resting membrane characterize Hb9⁺ INs. In most adjacent

Hb9⁻ INs, negative current injections produce hyperpolarization-dependent depolarization sags. (*Panel B* was used with permission from Hinckley and Ziskind-Conhaim, 2006).⁴⁰

Figure 3.

Cocktail-induced locomotor-like oscillations in synaptically isolated spinal cord are mediated by I_{NaP} . Simultaneous recording of neurochemically induced locomotor-like rhythms in Hb9 IN (*upper trace*) and electroneurograms of L1 or L2 ventral roots (*lower trace*). (**A₁**) The amplitude and frequency of induced CNQX-resistant voltage oscillations did not change after blocking glycinergic and GABAergic transmission (10 μ M CNQX + 15 μ M Picro + 0.5 μ M Str). Ventral root bursts were suppressed in the presence of CNQX. Action potentials are truncated. (**A₂**) Histogram of the amplitude and cycle period (mean $\pm$ SE, $n = 6$) as a function of blocking non-NMDARs (CNQX) or inhibiting both fast excitatory and inhibitory transmission (CNQX + Picro + Str). Disinhibition did not significantly change the amplitude CNQX-resistant membrane oscillations. Circular phase analysis demonstrates broadly distributed phase values, reflecting the lack of correlation between rhythmic activities in Hb9 INs and motoneurons in the synaptically isolated hemicord ($r = 0.12$). (**A₃**) In a different Hb9 IN, blocking nicotinic and muscarinic receptors

did not significantly alter the properties of membrane voltage oscillations in Hb9 INs. Nicotinic transmission was blocked with mecamylamine (Mec, 10 μ M) and dihydro-beta-erythroidine (DH β E, 50 μ M), and muscarinic receptors were blocked with atropine (Atrop, 10 μ M). Resting potentials varied between -52 and -56 mV. **(B)** Ril (10 μ M), a blocker of I^{NaP} , suppressed voltage oscillations in synaptically isolated Hb9 INs. Membrane potential was -50 mV. (Modified with permission from Ziskind-Conhaim *et al.*, 2008).³⁷

Figure 4.

Nickel but not mibefradil suppressed cocktail-induced voltage oscillations triggered by high concentrations of NMDA. (A) NMDA alone triggered membrane oscillations that were blocked after 20 min exposure to nickel. The inhibition was reversible within 30 min of nickel removal. Membrane potentials: -55 to -60 mV. B. In a different Hb9 IN, exposure to mibefradil did not block membrane voltage oscillations, but those were blocked by nickel. Membrane potentials: -53 to -57 mV. Action potentials are truncated.

Figure 5. Blocking BKCa channels suppressed the membrane oscillations generated by high-NMDA rhythmogenic cocktail. **(A)** Exposure to cocktail with low NMA (5 μ M) triggered voltage oscillations that became less regular but were not blocked in the presence of charybdotoxin. More regular pattern of voltage oscillations was apparent 20 min after removing charybdotoxin. Membrane potential: -52 to -55 mV. Action potentials are truncated. **(B)** In a different Hb9 IN, membrane oscillations triggered by high NMDA (20 μ M) cocktail were blocked within 21 min exposure to charybdotoxin. The inhibitory action was reversible. Membrane potential: -50 to -54 mV.

Figure 6.

Putative glutamatergic boutons from primary afferents on Hb9 interneurons. Stimulation of dorsal root afferents at two times threshold evoked short-latency EPSCs in a Hb9 IN. Top left: superimposed are three successive traces recorded at 0.1 Hz. Top right: expanded time scale to show that the onset of the evoked response (*white arrow*) was time-locked to the stimulus artifact (*triangle*), suggesting that these were monosynaptic EPSCs. The Hb9 IN was filled with neurobiotin (*red*). Combined immunohistochemistry against VGluT1 (*white*), parvalbumin (*blue*), and the Hb9 protein (*green*) revealed that proximal and distal dendrites receive putative primary afferent synapses (VGluT1⁺ and Parvalbumin⁺; both sets of four images acquired at the single optical level). The Hb9 IN was reconstructed from individually

acquired z -axis images and shown as a projection (main image; Hb9 shown in *red*). The soma of the neuron recorded from (neurobiotin) was positive for the Hb9 protein (set of three images at the bottom right), confirming its identity as a Hb9 IN.

Figure 7.

Putative contacts of Hb9 INs on motoneurons in the medial motor column. (A) Motoneurons were labeled with ChAT (*white*) to reveal the extent of somatic membrane. The image is a short z-stack projection from individual images. (B) The putative axon of a single-filled Hb9 interneuron. Neurobiotin was visualized with the ABC method and revealed with Rhodamine as the fluorochrome (*red*). (C) An overlay image from A and B. (D) Note the close apposition of three individual synaptic boutons from the Hb9 IN the soma of two medially located motoneurons.