Serotonin Modulates the Properties of Ascending Commissural Interneurons in the Neonatal Mouse Spinal Cord

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Zhong, Guisheng, Manuel Díaz-Ríos, and Ronald M. Harris-Warrick. Serotonin modulates the properties of ascending commissural interneurons in the neonatal mouse spinal cord. J Neurophysiol 95: 1545–1555, 2006. First published December 7, 2005; doi:10.1152/jn.01103.2005. The interneuron populations that constitute the central pattern generator (CPG) for locomotion in the mammalian spinal cord are not well understood. We studied the properties of a set of commissural interneurons whose axons cross and ascend in the contralateral cord (aCINs) in the studied the properties of a set of commissural interneurons whose axons cross and ascend in the contralateral cord (aCINs) in the neonatal mouse. During N-methyl-D-aspartate (NMDA) and 5-HT–induced fictive locomotion, a majority of lumbar (L2) aCINs examined were rhythmically active; most of them fired in phase with the ipsilateral motoneuron pool, but some fired in phase with contralateral motoneurons. 5-HT plays a critical role in enabling the locomotor CPG to function. We found that 5-HT increased the excitability of aCINs by depolarizing the membrane potential, reducing the postspike afterhyperpolarization amplitude, broadening the action potential, and decreasing the action potential threshold. Serotonin had no significant effect on the input resistance and sag amplitude of aCINs. These results support the hypothesis that aCINs play important roles in coordinating left–right movements during fictive locomotion and thus may be component neurons in the locomotor CPG in neonatal mice.

INTRODUCTION

Rhythmic movements such as locomotion and respiration are generated by local neural networks called central pattern generators (CPGs) (Marder and Calabrese 1996). The CPGs responsible for vertebrate locomotion are located in the spinal cord, producing basic motor output patterns for rhythmic and appropriately phased contractions of antagonistic muscle groups. The cellular organizations of CPGs for swimming in the lamprey and tadpole spinal cords have been well described (Grillner et al. 1998; Roberts et al. 1998). However, much less is known of the neuronal components of mammalian locomotor CPGs. Recent work has focused on the spinal cords of rats and mice, with a major focus on the lumbar spinal circuits controlling hindlimb movement (Butt et al. 2002; Kjaerulf and Kiehn 1996; Kremer and Lev-Tov 1997), although some work has been done in neonatal rats on the cervical spinal circuits controlling forelimb movement (Ballion et al. 2001; Juvin et al. 2005).

The commissural interneurons (CINs) are one class of interneurons thought to participate in the locomotion CPG: they send their axons across the midline and help coordinate left–right limb movements (Butt and Kiehn 2003; Butt et al. 2002). Four anatomically distinct types of CINs have been identified in the neonatal spinal cord preparations of rats and mice (Eide et al. 1999; Nissen et al. 2005); these neurons all send their axons across the midline and then extend their axons rostrally (ascending or aCIN), caudally (descending or dCIN), both rostrally and caudally (ascending and descending; adCIN) or remain locally within 1.5 segments of their somata (short-range CINs). CINs synapse on other interneurons and motoneurons on the opposite side of the spinal cord (Birinyi et al. 2003; Butt and Kiehn 2003), making them likely candidates to coordinate activity in the two sides during locomotion. More detailed physiological studies of dCINs have shown that they are indeed involved in left–right alternation in the neonatal rat (Butt and Kiehn 2003; Butt et al. 2002). The firing patterns of the dCINs are not homogeneous during N-methyl-D-aspartate (NMDA)- and serotonin-induced fictive locomotor-like activity; firing ranges from highly rhythmic bursting to dCINs that remain silent and do not participate in the motor pattern.

Although the function of aCINs in locomotion has been studied in other species (Buchanan 1999; Grillner et al. 1998; Matsuyama et al. 2004), there are no reports on the properties or activity of aCINs during fictive locomotion in the neonatal mouse. In this study, we begin to characterize the properties of aCINs in the neonatal mouse spinal cord. We studied their cellular firing properties and their activity during fictive locomotion, using whole cell patch recording from aCINs located in the L2 spinal segment and extracellular recording from the contralateral L2 ventral root. Fictive locomotor-like activity can be evoked in the isolated rodent spinal cord by a combination of NMDA and serotonin (Branchereau et al. 2000; Cazalets et al. 1992, 1995; Kjaerulf and Kiehn 1996), and serotonin is thought to be critical to organize the network for rhythmic activity (Christie and Whelan 2005; Liu and Jordan 2005; MacLean et al. 1998; Madriaga et al. 2004; Pearlstein et al. 2005). Our results show that a majority of aCINs fire rhythmically during fictive locomotion. To begin to understand how these neurons might contribute to CPG function, we studied their intrinsic membrane properties in both whole cord and slice preparations. In invertebrate and vertebrate CPGs, serotonin and other neuromodulators reconfigure the motor patterns at least in part by modifying the firing properties of the component neurons (Ayali and Harris-Warrick 1999; Harris-Warrick et al. 1998; Marder 2000; Stein et al. 1997). We thus studied how serotonin alters the intrinsic membrane properties and excitability of aCINs. Our results demonstrate that serotonin increases the excitability of aCINs and changes their intrinsic firing and spike properties. These results have been previously published in abstract form (Díaz-Ríos et al. 2005).
METHODS

Whole cord preparations

Experiments were performed using spinal cords of 0- to 5-day-old (P0–P5) ICR mice (Taconic, Hudson, NY). The animal protocol was approved by the Animal Use and Care Committee at Cornell University and was in accordance with National Institutes of Health guidelines. Animals were killed by decapitation, and their spinal cords were isolated by ventral laminectomy under ice-cold (4°C) oxygenated (95% O2-5% CO2) low-calcium Ringer 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 from segments C4 to S1 was removed and pinned ventral side-up and superfused with oxygenated normal Ringer solution composed of (in mM) 111 NaCl, 3.08 KCl, 25 NaHCO3, 1.18 KH2PO4, 1.25 MgSO4, 2.52 CaCl2, and 11 d-glucose. Experiments were performed after incubating preparations in oxygenated regular Ringer solution at room temperature (20–23°C) for 1 h.

To make whole cell recordings from the intact cord, a patch electrode was lowered into a small slit in the ventromedial surface of the L2 segment. Electrodes were pulled from thick wall borosilicate glass (WPI, Sarasota, FL) on a vertical puller (Narishige) and had resistances of 6–8 MΩ. The pipette solution contained (in mM) 138 K-glucolate, 10 HEPES, 0.3 ATP-Mg, and 0.0001 CaCl2 (pH 7.4 with KOH). The seal resistance obtained before recordings was always >1 GΩ. Blind patch-clamp recordings were made from the soma with a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA). Experiments were driven by Clampex (pClamp 9, Axon Instruments), and data were filtered at 2 kHz and digitized at 5 or 10 kHz.

To identify the CINs, the hemicord was slit down the midline rostrally between T13 and L1 and caudally between L4 and L5 for several segments before sectioning mid-sagittally on the contralateral side, thus liberating contralateral hemiscols several segments rostral and caudal of the recording site (Fig. 1A). The rostral and caudal hemicords contralateral to the site of the recording were gently sucked into stimulation electrodes. Brief current pulses (50–300 μA) were delivered through stimulation-isolation units to stimulate axons that were rostral and rostral or caudal to the recorded neuron (Fig. 1A). adCINs were conditionally identified if rostral but not caudal hemicord stimulation evoked an antidromic action potential. To verify the identity of the aCINs, two steps were taken. First, collision experiments were performed, where orthodromic action potentials were elicited by intracellular current injection while antidromic action potentials were delivered from the contralateral hemicord. By varying the time delay between orthodromic and antidromic stimulation, a delay was obtained when the action potentials collided and the antidromic action potential disappeared from the intracellular recording. Second, the collision experiments were repeated in the presence of 10 μM CNQX, 15 μM AP-5, 5 μM picrotoxin, and 10 μM strychnine to block fast synaptic transmission. In our preparations, these blockers were sufficient to eliminate nearly all detectable fast synaptic transmission. Neurons were accepted as aCINs if they had antidromic action potentials from the contralateral rostral hemiscol which collided with orthodromic action potentials from the soma, but which failed on these tests for caudal hemicord stimulation. We have completed a separate study of adCINs and found that they have markedly different properties from aCINs (unpublished observations), so we are confident that our aCIN population is not contaminated with adCINs.

Locomotor-like activity was evoked by perfusion with mouse Ringer solution containing a combination of NMDA (3–10 μM) and 5-HT (6–15 μM). A small-diameter suction electrode was placed on the L4 root contralateral to the recorded neuron to monitor flexor activity in the motor pattern; in a couple of experiments, the contralateral L5 root (cL5) was monitored to record contralateral extensor activity by limiting contralateral hemicord stimulation electrode to the caudal end of cL5 (which is in phase with ipsilateral L4 flexor activity). Ventral root recordings were band-pass-filtered (100 Hz to 1 kHz) and recorded using an AC amplifier (Model 1600 from A-M systems). All drugs were purchased from Sigma (St. Louis, MO): d(-)2-amino-5-phosphonopentanoic acid (AP-5), 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX), strychnine, picrotoxin, carbamoxalone, and TTX.
Slice preparations

Spinal cords were isolated in low-calcium Ringer solution. CINs were labeled retrogradely by making fine slits in the contralateral hemiscot rostrally (T1–L1) and caudally (L1–L5) of the target L1–L2 regions, and applying crystals of fluorescent dextran amines (3,000 MW Texas red dextran amine, and 3,000 MW fluorescein dextran amine; Molecular Probes, Eugene, OR) to the slits (Fig. 1C), as described previously (Glover 1995). Preparations were incubated in oxygenated mouse Ringer at 30°C for 1–2 h to let the dyes diffuse to the cell bodies. Transverse spinal cord slices (250–350 μm) were made with a vibrating microtome (Leica Microsystems) and transferred to regular mouse Ringer solution at 30°C for 30–45 min before recording at room temperature (20–23°C) with constant perfusion (3 ml/min) of mouse Ringer. Patch-clamp electrode resistance and contents were the same as for whole cord recordings. Fluorescein- and rhodamine-labeled neurons contralateral to the dye injection sites were identified under epifluorescent illumination and visualized using differential interference contrast (DIC) optics (Axioskop 2FS plus, Carl Zeiss). Drugs were added by perfusion as described above.

Data analysis

Locomotor-like activity was recorded in the intact spinal cord preparation during bath application of NMDA and 5-HT. Clampfit 9.0, Excel, and Spike 2 were used for data analysis. A cycle of motor nerve activity started at the onset of a burst of the contralateral L2 (cL2) ventral root and ended at the onset of the next cL2 burst. We used a double normalization process to analyze the phasing of neural activity during the locomotor cycle (Berkowitz and Stein 1994; Shefchyk and Jordan 1985). The period of cL2 activity was divided into first five equal time bins, while the rest of the cycle was also divided into five equal time bins. The mean action potential firing rate in each bin was established by dividing the number of action potentials by the bin duration for ≥35 cycles of activity. The average firing frequencies in each bin were used to create an activity histogram (Fig. 2B).

Circular statistics were used to determine the significance of the phasing of aCIN firing during fictive locomotion (Fig. 2C). The latency to each spike was measured relative to the beginning of the contralateral L2 ventral root burst. The mean latency was calculated as described by Kjaerulff and Kiehn (1996). The direction of a vector represents the average preferred phase of firing of the neuron and its length, r, indicates the tuning of the spikes around their mean. Because dual-reference analysis was used in this study to minimize the effects of variability in the duty cycle, vectors with a phase in the range of 0.0–0.5 corresponded to cells firing in phase with iL2. P values for the significance of r were calculated as described by Zar (2000) and Kjaerulff and Kiehn (1996).

Firing properties of isolated aCINs

aCINs were isolated from rapid synaptic inputs with a combination of AP-5, CNQX, picrotoxin, and strychnine as described above. Some of the neurons fired spontaneously, whereas others were silent at rest and required depolarizing current pulses to evoke action potentials. To assure uniformity of the measurements, all neurons were held at −60 mV with a bias current. Action potentials with peak amplitude above 0 mV were included in the analysis. To determine the F-I plot, 1-s current injections of increasing amplitude were delivered, and the average spike frequency during a step was determined by counting the number of spikes during the 1-s step, and plotted against the injected current amplitude. The slope of the F-I curve was linearly fitted. Spike adaptation was quantified as the ratio of the average of the last three spike frequencies compared with the first three spike frequencies in a 1-s train (Fig. 3D). The voltage threshold for action potential generation was measured as the peak of the second derivative of voltage with time at the beginning of the action potential. Spike afterhyperpolarization (AHP) amplitude was measured from the action potential threshold to the minimal voltage after the action potential. Additionally, we also measured the peak AHP, the minimal voltage after the action potential. The action potential half-width was established at the voltage that is halfway from the threshold to overshoot. The hyperpolarization-activated sag depolarization was determined with 1-s hyperpolarizing steps of increasing current amplitude to generate minimal voltages down to −120 mV. The sag voltage was measured from the minimal membrane potential near the beginning of the step to the voltage at the end of the step. On release of the hyperpolarizing steps, the neurons often rebounded with a small depolarization, which was measured relative to the holding potential (typically −60 mV). Input resistance was estimated by applying small hyperpolarizing current pulses. In our whole cord and slice preparations, the intrinsic membrane properties of aCINs and their responses to serotonin are comparable. Thus we pooled the data from whole cord and slice preparations. The effects of serotonin on the membrane properties were analyzed by paired two-tailed Student’s t-test. Results were
trails to traverse the absolute refractory distance after the collision in the axon, causing the antidromic spike to fail as it propagates through the cell, and varying the time between ortho- and antidromic spikes by current injection into the cell. Second, we performed a spike collision test by stimulating the caudal (L4–L5) contralateral hemicord. In whole cord preparations, all patch recordings were made in the L2 ventromedial area, which contains critical components of the hindlimb locomotor CPG (Kjaerulff and Kiehn 1996; Kremer and Lev-Tov 1997). During these experiments, we recorded from 210 neurons in this area: 66 were identified as CINs, with 29 aCINs, 27 dCINs, and 10 adCINs.

To label aCINs in transverse slices of the spinal cord, slits were made in the rostral and caudal contralateral hemispheres of the intact preparation, and crystals of fluorescent dextran amine (caudal) or Texas red dextran amine (rostral) were placed in the slits. After incubation for 1–2 h to allow the dyes to retrogradely transport to the somata, slices were prepared. Interneurons in the contralateral ventromedial region that were stained by Texas red but not by fluorescein were identified as aCINs (Fig. 1C). As described by Eide et al. (1999), there was a small possibility of false identification if some of the axons did not label adequately; however, the results with aCINs recorded in the slice were very similar to those identified electrophysiologically in the whole cord, so we do not think this was a significant problem in our results. Seventy-eight percent of aCINs (n = 37: 27 from whole cord preparations and 10 from slice preparations) we recorded were silent when most synaptic transmission was blocked, with stable average resting membrane potentials of −51.5 ± 3.4 mV (n = 29). The remaining aCINs (8/37) displayed spontaneous firing, with an average frequency of 3.5 ± 0.4 Hz.

**Firing properties of aCINs during 5-HT- and NMDA-induced fictive locomotion**

The first question we addressed, using the whole cord preparations, was whether aCINs fire rhythmically during fictive locomotion and thus might be components of the CPG for locomotion. We usually monitored locomotor-like activity by recording the contralateral L2 ventral root; in a few cases, contralateral L5 activity was also recorded (Fig. 2A). Fictive locomotor-like activity was induced by superfusion of a combination of 5-HT and NMDA (Fig. 2A), which activated a pattern of left–right ventral root burst alternation, as well as ipsilateral alternation between the L2 (flexor-dominated) and L5 (extensor-dominated) ventral roots. Figure 2, A–C, shows an example of an aCIN firing during NMDA- and 5-HT–induced locomotor-like activity. Data were analyzed only in preparations that showed stable rhythmic activity in cL2 and where the aCIN’s action potential overshoot was >0 mV. Ten aCINs met these criteria. Individual aCINs were recorded for ≈15 min during NMDA- and 5-HT–induced locomotor-like activity. To determine the preferred firing phases of aCINs, the phase of each spike during ≈35 cycles was normalized by the onset of the cL5 burst (primarily extensor-related, and thus in phase with the ipsilateral flexor L2 burst). A double-normalization process was used (Berkowitz and Stein 1994; Shefchyk and Jordan 1985), where the normalized cycle periods were subdivided into 10 bins, the first 5 representing the phase of iL2 (or cL2) bursting and the last 5 equivalent to the cL5 phase (see

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**Identification of aCINs**

We used two preparations to study the properties of aCINs in the neonatal mouse spinal cord, the intact cord, and 250- to 350-μm transverse slices from the upper lumbar region (L1–L2). In the intact spinal cord, aCINs were identified by the presence of antidromic action potentials on stimulation of the caudal (T13–L1) but not the spinal (L4–L5) contralateral hemisegment. Two steps were taken to confirm that the action potentials arose from antidromic axon stimulation and not from synaptic drive. First, fast synaptic transmission was blocked with a combination of 5-HT and NMDA (Fig. 2A), which activated a pattern of left–right ventral root burst alternation, as well as ipsilateral alternation between the L2 (flexor-dominated) and L5 (extensor-dominated) ventral roots. Figure 2, A–C, shows an example of an aCIN firing during NMDA- and 5-HT–induced locomotor-like activity. Data were analyzed only in preparations that showed stable rhythmic activity in cL2 and where the aCIN’s action potential overshoot was >0 mV. Ten aCINs met these criteria. Individual aCINs were recorded for ≈15 min during NMDA- and 5-HT–induced locomotor-like activity. To determine the preferred firing phases of aCINs, the phase of each spike during ≈35 cycles was normalized by the onset of the cL5 burst (primarily extensor-related, and thus in phase with the ipsilateral flexor L2 burst). A double-normalization process was used (Berkowitz and Stein 1994; Shefchyk and Jordan 1985), where the normalized cycle periods were subdivided into 10 bins, the first 5 representing the phase of iL2 (or cL2) bursting and the last 5 equivalent to the cL5 phase (see...
aCINs were defined as rhythmic if $P < 0.05$ by the Rayleigh test (Zar 2000). aCINs did not show homogeneous firing patterns during fictive locomotion. Eight of the 10 aCINs depolarized and showed significantly rhythmic discharge ($P < 0.05$, Rayleigh test). aCINs did not show homogeneous distributions, so we pooled the data from both preparations in the following analysis. The mean capacitance of aCINs was 75.5 ± 31.8 pF ($n = 37$), whereas the mean input resistance was 644.1 ± 276.7 MΩ ($n = 37$). We characterized the input–output properties of aCINs by injecting a series of increasing depolarizing current steps and recording the spike activity of the neurons ($n = 37$; Fig. 3A). The average spike frequency and the instantaneous first spike frequency were plotted as a function of the injected current ($F-I$ plots; Fig. 3, B and C). The average $F-I$ slope was $122 ± 23$ Hz/nA ($n = 37$, Fig. 3B) over a range of current injections. Spike adaptation, which plays an important role in many CPGs (Marder 2000; Marder and Calabrese 1996), was clearly present, as seen by the higher $F-I$ slope for the first spike pairs in each step (201 Hz/nA; Fig. 3C). We quantified the spike adaptation as the average of the last three spike frequencies over the first three spike frequencies in the train (Fig. 3D). aCINs showed moderate spike adaptation ($1.16 ± 0.14$ at 10 Hz), which was frequency dependent (Fig. 3E; $n = 37$).

Subthreshold responses of aCINs

The hyperpolarization-activated inward current, $I_h$, is often found in neurons participating in rhythmic neural networks (Harris-Warrick et al. 1998; Mironov et al. 2000). This can be detected in current clamp by a depolarizing sag voltage during a maintained hyperpolarizing current injection. We measured the sag voltage in aCINs as the voltage difference between the minimum voltage and the voltage at the end of a 1-s hyperpolarizing current step from a resting potential of $-60$ mV (Fig. 4A). The slow deactivation of $I_h$ also causes a depolarizing rebound above the holding potential at the end of the hyperpolarizing current step (Fig. 4A). Seventy-six percent of aCINs (28/37) had detectable sags and rebound potentials. These responses grew with increasing hyperpolarizations (Fig. 4B). A typical $I-V$ curve for the sag voltage of an aCIN is shown in Fig. 4C. When measured during a hyperpolarizing step to a minimum voltage of $-120$ mV, the average sag amplitude was $8.3 ± 4.5$ mV, whereas the average rebound potential was $3.4 ± 2.2$ mV. In three aCINs, the rebound potential passed the threshold of action potential and generated spikes; this depended on the duration of the hyperpolarizing step (Fig. 4D) and on the hyperpolarizing voltage dependent activation of $I_h$ (Fig. 4E). As expected, the sag and rebound potentials were correlated with each other (Fig. 4F), supporting the hypothesis that the rebound potential is caused by $I_h$, which generates the voltage sag in these aCINs.

Effect of 5-HT on membrane potential

Serotonin plays a critical role in the generation of locomotion in the neonatal rodent spinal cord (Christie and Whelan 2005; Liu and Jordan 2005; MacLean et al. 1998; Madriaga et al. 2004). We hypothesized that it acts in part to modulate the intrinsic properties of CPG neurons, including the commissural interneurons, to enable the locomotor network to function. Thus our next aim was to study the effects of 5-HT on the intrinsic membrane properties of aCINs

To understand how aCINs might function within the locomotor CPG, it is necessary to understand their intrinsic membrane properties. We isolated aCINs from most rapid synaptic inputs using the blocker Ringer described above; in this solution, spontaneous synaptic inputs were rarely if ever seen. The intrinsic properties of aCINs isolated in this way were indistinguishable between the intact spinal cord and slice preparations, so we pooled the data from both preparations in the following analysis. The mean capacitance of aCINs was 75.5 ± 31.8 pF ($n = 37$), whereas the mean input resistance was 644.1 ± 276.7 MΩ ($n = 37$). We characterized the input–output properties of aCINs by injecting a series of increasing depolarizing current steps and recording the spike activity of the neurons ($n = 37$; Fig. 3A). The average spike frequency and the instantaneous first spike frequency were plotted as a function of the injected current ($F-I$ plots; Fig. 3, B and C). The average $F-I$ slope was $122 ± 23$ Hz/nA ($n = 37$, Fig. 3B) over a range of current injections. Spike adaptation, which plays an important role in many CPGs (Marder 2000; Marder and Calabrese 1996), was clearly present, as seen by the higher $F-I$ slope for the first spike pairs in each step (201 Hz/nA; Fig. 3C). We quantified the spike adaptation as the average of the last three spike frequencies over the first three spike frequencies in the train (Fig. 3D). aCINs showed moderate spike adaptation ($1.16 ± 0.14$ at 10 Hz), which was frequency dependent (Fig. 3E; $n = 37$).

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membrane properties of the aCINs. As described earlier, some aCINs fired spontaneously at rest, whereas the others were silent. To allow more uniformity in the measurements, the membrane potential of all cells was adjusted to \(-100\) mV to remove spontaneous firing. Because the range of 5-HT concentrations that evoked fictive locomotion was from 6 to 15 \(\mu\)M, we measured the effects of 9 \(\mu\)M 5-HT on the aCINs. In normal Ringer solution, 5-HT (9 \(\mu\)M) depolarized the membrane potential of aCINs (Fig. 5A) and, in some cells, this depolarization exceeded the threshold for action potential generation. When no bias current was injected, aCINs were much more likely to start firing action potentials in 5-HT application. To verify that the depolarization is a direct action of serotonin on the aCINs, blockers of fast synaptic transmission were added to the Ringer solution. Under this condition, 5-HT still depolarized the membrane potential (Fig. 5B) and often evoked action potentials in both whole cord and slice preparations (Fig. 5C, 1 example from the whole cord preparation). The average depolarization caused by 9 \(\mu\)M 5-HT was 9.2 \(\pm\) 2.5 mV with most fast synaptic transmission blocked (\(n = 27\)). All further experiments were performed in the presence of blockers of fast synaptic transmission, to look for direct effects of 5-HT on these neurons.

Membrane oscillations induced by 5-HT

Interestingly, in about one-half of the aCINs (13/27), serotonin evoked relatively regular small rhythmic voltage oscillations when fast synaptic transmission was blocked. The mean frequency of oscillations was 3.3 \(\pm\) 0.5 Hz, and their amplitude was 3.5 \(\pm\) 0.8 mV. On removal of 5-HT, this effect was totally reversible (Fig. 5, C and D). These oscillations did not seem to arise from fast glutamatergic synaptic transmission because they persisted in the blocking solution. To further identify possible mechanisms contributing to these oscillations, we applied TTX (1 \(\mu\)M) to block action potentials in slice preparations; this completely and reversibly abolished the small oscillations (Fig. 6A; \(n = 3\)). We also tested carbenoxolone (100 \(\mu\)M), a gap junction blocker; carbenoxolone also blocked the 5-HT–induced oscillations (Fig. 6B; \(n = 3\)). This effect was reversible, but a long time (\(\approx 1\) h) was needed for a complete carbenoxolone washout. Finally, the amplitude of the 5-HT–evoked oscillations did not change significantly when hyperpolarizing currents were injected to hyperpolarize aCINs as far as \(-85\) mV (Fig. 6C; \(n = 5\); 3 from slice preparations, 2 from whole cord preparations). Our results suggest that these oscillations may arise from action potentials in unidentified neurons.

![FIG. 5. Serotonin depolarizes aCINs and induces low frequency membrane oscillations. A: 5-HT (9 \(\mu\)M) reversibly depolarized the membrane potential of an aCIN; in the absence of blockers of synaptic transmission, a significant increase in synaptic drive was seen. B: 5-HT (9 \(\mu\)M) reversibly depolarized the membrane potential of an aCIN with most fast synaptic transmission blocked. C: in some neurons, 5-HT (9 \(\mu\)M) induced low-amplitude membrane oscillations with most fast synaptic transmission blocked. D: traces were amplified from C showing aCIN response before, during, and after 5-HT application.](http://jn.physiology.org/)

![FIG. 6. Serotonin induces TTX- and carbenoxolone-sensitive subthreshold oscillations in the membrane potential of aCINs in slice preparations. A: TTX (1 \(\mu\)M) blocked the oscillations caused by 5-HT (9 \(\mu\)M) application with most fast synaptic transmission blocked. B: carbenoxolone (100 \(\mu\)M), a gap junction blocker, blocked the oscillation caused by 5-HT (9 \(\mu\)M) application with most fast synaptic transmission blocked. C: effects of hyperpolarization on oscillation caused by 5-HT (9 \(\mu\)M) application with most fast synaptic transmission blocked.](http://jn.physiology.org/)
that are electrically coupled to aCINs through carbenoxolone-sensitive gap junctions.

**Effect of 5-HT on the excitability of aCINs**

The effects of 5-HT on the excitability of aCINs were studied with major fast synaptic transmission blocked. Holding currents were adjusted to keep the membrane potential at $-60$ mV in different conditions. First, the $F-I$ relation was determined with a series of increasing depolarizing currents in control conditions and during serotonin application. Figure 7A shows the responses of one aCIN in control, during serotonin application, and after washout of the amine. At its normal resting potential, bath application of 5-HT (9 $\mu$M) depolarized this aCIN by 9.5 mV; for the $F-I$ analysis, the cell was held at $-60$ mV with hyperpolarizing bias current. 5-HT significantly reduced the current threshold for spike generation, with a reduction in rheobase from 20 to 15 pA. The average spike frequency was plotted in an $F-I$ plot (Fig. 7B): the control $F-I$ plot was linear, with a slope of 120 Hz/nA. 5-HT (9 $\mu$M) shifted the $F-I$ plot to the left, but did not change the slope. The effects of 5-HT were reversible after a 10-min wash (Fig. 7, A and B).

Similar effects of 5-HT on the $F-I$ relationship were observed in 19 other aCINs examined: the plot was shifted up but without a change in slope, so that more action potentials were generated by each current step (Fig. 7B and C). To quantify the effect of 5-HT on spike frequency, current steps generating approximately four action potentials under control conditions were compared under control and 5-HT conditions. Serotonin increased the number of action potentials per pulse by 80% in both the whole cord and slice preparations (control: 4.2 ± 1.1 Hz; 5-HT: 7.6 ± 2.8 Hz; washout: 4.9 ± 1.7 Hz; n = 20, P < 0.0001: Fig. 7C). At the same time, the rheobase was decreased by 33% from 45 ± 15 to 30 ± 12 pA (P < 0.001, n = 14). Collectively, the $F-I$ slope under control conditions was 160 ± 6 Hz/nA; during 5-HT application, it did not change significantly (173 ± 15 Hz/nA; n = 20; P > 0.1). After a 10-min wash, the slope recovered to 170 ± 18 Hz/nA (n = 20; P > 0.1 vs. control, paired t-test).

The effects of 5-HT on spike adaptation were also characterized. Because we found that the degree of spike adaptation was strongly dependent on the average firing frequency (Fig. 3E), we compared control and 5-HT effects on spike adaptation when the spike frequency was adjusted to 10 Hz in the control, 5-HT application, and washout conditions; this required comparing responses to different amplitude current steps (Fig. 8A). When the effects of 5-HT on spike frequency were removed in this fashion, we did not find any changes in spike adaptation with 5-HT (control: 1.17 ± 0.09; 5-HT: 1.15 ± 0.1; washout: 1.16 ± 0.1; P > 0.1; n = 20: Fig. 8B).

5-HT has little effect on input resistance, sag, and rebound potential

One possible way for 5-HT to enhance neuronal excitability to current injection is to increase the input resistance (e.g., by inactivating potassium currents) (Perrier et al. 2003). To test
this possibility, we measured the initial aCIN voltage response to hyperpolarizing currents (Fig. 9A). 5-HT had a trend to modestly increase the input resistance of aCINs, but this effect was not significant (control: 644 ± 276 MΩ; 5-HT: 671 ± 316 MΩ; washout: 656 ± 289 MΩ; P = 0.08; n = 20; Fig. 9, B and C). Serotonin did not obviously alter the mean sag voltage evoked by hyperpolarizing current steps (Fig. 9, A and D). To quantify the effect of serotonin on sag amplitude and rebound potential, we measured these parameters after a current step to a minimum potential of about −120 mV. There was no significant effect of 5-HT on the sag (control: 8.6 ± 4.3 mV; 5-HT: 7.8 ± 3.7 mV; washout: 8.4 ± 3.9 mV; P > 0.05; n = 20). The rebound potential after the end of the current step was also unaffected (control: 4.6 ± 3.8 mV; 5-HT: 4.1 ± 3.4 mV; washout: 3.9 ± 3.4 mV; P = 0.12, n = 20).

**Effects of 5-HT on AHP and spike threshold**

Serotonin affected the shape of the action potentials firing during a depolarizing current step (Fig. 10A). To test whether 5-HT had any effects on action potential parameters, we compared these parameters measured from the action potentials firing at low frequency (~3 Hz, Fig. 10A), because the AHP amplitude, the threshold, and half-width of action potentials are all dependent on the firing frequency. The voltage threshold for action potential generation was measured as the peak of the second derivative of voltage with time and is labeled by the upper dashed lines in Fig. 10A. As expected from the reduced rheobase measurements, serotonin decreased the action potential threshold significantly (control: −30.5 ± 7.5 mV; 5-HT: −32.1 ± 8.3 mV; washout: −30.8 ± 6.9 mV; P = 0.003; n = 20). The postspike AHP affects neuronal excitability and maximum spike frequency. Our results showed that serotonin significantly reduced the amplitude of the AHP by 14% (control: 18.3 ± 5.5 mV; 5-HT: 15.7 ± 4.7 mV; washout: 17.9 ± 4.7 mV; P < 0.001; n = 20; Fig. 10B), but not the time to AHP peak, measured from the AP threshold downstroke to the minimum potential after one action potential (control: 33 ± 10 ms; 5-HT: 36 ± 10 ms; washout: 32 ± 10 ms; P = 0.2, n = 20; Fig. 10C). To assure the effects of 5-HT on AHP amplitude are not caused by a decrease in AP threshold, we compared the peak AHP during control, 5-HT, and washout conditions. As shown in Fig. 10A, 5-HT significantly reduced the peak AHP (control: −48.7 ± 4.4 mV; 5-HT: −46.9 ± 5.5 mV; washout: −48.0 ± 5.1 mV; n = 20; P < 0.001). Finally, we found that serotonin significantly broadened the AP width at half-peak amplitude (control: 3.1 ± 0.6 ms; 5-HT: 3.6 ± 0.7 ms; washout: 3.4 ± 0.7 ms; P = 0.015, n = 20; Fig. 10, D and E).
DISCUSSION

Possible function of aCINs in fictive locomotion

We studied a set of aCINs located in the ventromedial area of the lumbar spinal cord. Lesion and labeling studies have shown that the rodent spinal CPG for hindlimb locomotion is broadly distributed in this region between the caudal thoracic and the end of the lumbar cord (Cazalets et al. 1995; Kiehn and Kjaerulff 1998; Kjaerulff and Kiehn 1996). When the cord is split longitudinally, each hemicord can generate its own locomotor rhythm (Bonnot and Morin 1998; Branchereau et al. 2000). In the intact cord, commissural interneurons normally coordinate these independent oscillators to generate left/right alternation in many species, including lamprey (Buchanan 1999; Buchanan and McPherson 1995), Xenopus tadpoles (Soffe et al. 1984), fetal (Nakayama et al. 2002) and neonatal rats (Butt and Kiehn 2003; Butt et al. 2002), and cats (Matsuyama et al. 2004). CINs are heterogeneous in their effects, with both glutamatergic and glycinergic CINs providing contralateral excitation and inhibition, respectively (Butt and Kiehn 2003). In the neonatal rat spinal cord, motoneurons receive both mono- and polysynaptic excitatory and inhibitory inputs from contralateral CINs in phase with the fictive motor pattern (Birinyi et al. 2003; Butt et al. 2002). In swimming vertebrates, such as lamprey (Buchanan 1999; Buchanan and McPherson 1995) and Xenopus tadpoles (Soffe et al. 1984), commissural interneurons fire one or a few spikes at a specific phase to inhibit the contralateral oscillator and motoneurons; this simple reciprocal inhibition strategy assures left–right alternation for normal swimming (Buchanan 1999; Buchanan and McPherson 1995; Soffe et al. 1984). However, the locomotor pattern in tetrapod vertebrates is considerably more complex, with multiple muscles firing at different phases on each side, so the function of the CINs must be more complicated than in swimming vertebrates.

Our results provide information about the function of aCINs during fictive locomotion in neonatal mice. The large majority of aCINs we examined fired rhythmically during fictive locomotion, consistent with a role in the locomotor CPG. aCINs typically fired multiple spikes per burst, as was also found with dCINs in the neonatal rat (Butt and Kiehn 2003; Butt et al. 2002) and in cats (Matsuyama et al. 2004). As was previously shown for rat dCINs (Butt et al. 2002), the peak of aCIN activity occurs over a much wider range of phases during fictive locomotion than in the swimming vertebrates; 62.5% of rhythmically active aCINs fired at some point during the ipsilateral L2 flexor motoneuron burst, but the peak of activity could be at the beginning of the ipsilateral phase in some neurons and at the end in others; in addition, 37.5% of aCINs fired in phase with contralateral flexors, which is in phase with ipsilateral extensor motoneurons. This complex firing pattern suggests a sophisticated controlling mechanism to mediate both left/right and flexor/extensor alternation.

Our experiments support the idea that aCINs are involved in the coordination of mouse locomotion. A subset of CINs can be identified by expression of the transcription factor Dbx1 during development; a majority of these neurons are excitatory and inhibitory aCINs (Pierani et al. 2001). Mutant mice lacking Dbx1 have lost these CINs and display aberrant left–right coordination with periods of synchronized activity, although normal ipsilateral flexor–extensor alternation occurs and there seems to be some homeostatic reorganization in the knock-out mice (Lanuz et al. 2004). These experiments further suggest that aCINs are likely to be involved in fictive locomotion in neonatal rodents.

Based on the firing pattern of aCINs during fictive locomotion, we can start to speculate on their possible roles in locomotion. Three possibilities can be envisaged. First, aCINs may play critical roles in coordinating hindlimb left–right alternation. As stated above, the hindlimb CPG is distributed from lower thoracic segments (T12–T13) to lower lumbar segments (L5–L6) (Kiehn and Kjaerulff 1998; Kjaerulff and Kiehn 1996), and aCINs could coordinate activity in this multisegmental distributed hindlimb CPG. Supporting this hypothesis, the Dbx-1 knock-out mice show a marked disruption of left–right alternation during fictive locomotion (Pierani et al. 2001). Second, aCINs in the hindlimb region may send axons rostrally to coordinate movements of the forelimbs with the hindlimbs. The forelimb CPG is distributed approximately between cervical segment C7 and thoracic segment T1, and aCINs may be involved in this coordination. Anatomical tracing studies have shown that lumbar aCINs can project anteriorly to at least six segments (Birinyi et al. 2003; Eide et al. 1999), but whether they project more rostrally than this is unknown. Thus this coordination between hind- and fore-limbs may be polysynaptic. Third, aCINs may provide an efference copy of the motor pattern to brain stem and higher circuitry that drive locomotion. Again, this may be a polysynaptic pathway.

Modulation of aCINs by serotonin

Serotonin seems to play a central role in enabling the CPG for locomotion in the neonatal rodent (Christie and Whelan 2005; Liu and Jordan 2005; MacLean et al. 1999; Madriaga et al. 2004; Pearlstein et al. 2005). Both ketanserin (a 5-HT2 receptor antagonist) and SB 269970 (a 5-HT7 receptor antagonist) disrupt the locomotor-like activity induced by electrical stimulation from brain stem in neonatal rodent spinal cord, suggestive of the contribution of endogenous 5-HT in rodent locomotion (Liu and Jordan 2005; Pearlstein et al. 2005). While this suggests that 5-HT will have a strong effect on the rhythm-generating kernel, it was not obvious that 5-HT would also affect the CINs, which could in theory passively transfer the information from the excited half-center oscillator to the opposite side of the cord. However, our results show that 5-HT does indeed directly excite the aCINs. 5-HT significantly depolarizes aCINs even when most rapid glutamatergic, GABAergic, and glycinergic synaptic transmissions are blocked. 5-HT also decreases the voltage threshold for action potential generation and decreases the rheobase, making the neurons more excitable to synaptic inputs. The amplitude of the AHP is reduced, and the spike half-width is increased. Collectively, these effects of 5-HT all contribute to the increase of excitability of aCINs in the neonatal mouse spinal cord. Serotonin’s ability to broaden the spike half-width is also found in locust motorneurons (Parker 1995). If such an effect occurred at presynaptic nerve terminals, it would allow more calcium influx in response to the action potential, and could enhance aCIN synaptic strength.
Our experiments have not yet addressed the ionic mechanisms by which 5-HT excites aCINs. However, the reduction in AHP amplitude and peak AHP, increase in spike width, and reduction in spike threshold, along with a trend to increase the input resistance, could all be explained at least in part by a reduction in a calcium-activated potassium current. In rodent motoneurons (Gao and Ziskind-Conhaim 1998), \( I_{\text{K(Ca)}} \) plays a significant role in the slow AHP; in dCINs, this is mediated by an apamin-sensitive current, probably the SK-type potassium channel (R.H.-W., unpublished results). Because the AHP is believed to contribute to spike adaptation, we looked for an effect of 5-HT on this parameter; however, after correction for 5-HT effects on spike frequency, there is no change in spike adaptation, suggesting that other ion currents, such as fast inactivating sodium currents, could also be modulated by 5-HT (Miles et al. 2005). The slow hyperpolarization-induced sag depolarization, and the consequent depolarizing rebound potential were not significantly affected by 5-HT; these are usually caused by the activation of \( I_h \) (Kjaerulff and Kiehn 2001). Other species, using a variety of different mechanisms. In lamprey, serotonin slows fictive locomotion and greatly strengthens the firing rate of motoneurons (Harris-Warrick and Cohen 1985). In part this arises from a reduction in the late phase of AHP without changing the basic shape of action potentials of motoneurons and interneurons in the in vitro preparations (Christenson et al. 1989). This effect of 5-HT on AHP is caused by inhibition of calcium-activated potassium currents (El Manira et al. 1997; Hill et al. 2003). The effect of 5-HT on the membrane properties of motoneurons have been studied intensively in many species (Hsiao et al. 1997; Kjaerulff and Kiehn 2001; Perrier et al. 2003). 5-HT generally increases the excitability of motoneurons, using many different ionic mechanisms. In the neonatal rat, 5-HT reduces the threshold for action potential generation in motoneurons (Gilmore and Fedirchuk 2004) and unidentified ventral horn neurons (Fedirchuk and Dai 2004). The postspike AHP amplitude is reduced by the application of 5-HT, in part by an enhancement of \( I_h \) (Hsiao et al. 1997; Kjaerulff and Kiehn 2001) and an increase in a low-threshold \( I_{\text{Ca}} \) (Berger and Takahashi 1990). Similar effects are seen on hypoglossal motor neurons (Berger et al. 1992). 5-HT also directly depolarizes neonatal rat phrenic motoneurons (Lindsay and Feldman 1993) and cat motoneurons (White and Fung 1989).

Electrical synapses in fictive locomotion

Tresco and Kiehn (2000) showed that, in the neonatal rat spinal cord, motoneurons are electrically coupled strongly enough to show coordinated local oscillations in the presence of TTX when NMDA and 5-HT are added, although different motor pools were not coordinated. Carbenoxolone, which blocks electrical synapses, abolished the coordination between motoneuron oscillations (Tresco and Kiehn 2000). Gap junctions are expressed in both motoneurons and interneurons in the neonatal rat spinal cord (Chang et al. 1999) and could play a role to synchronize firing of sets of neurons (Sohl et al. 2005). Our electrophysiological studies suggest that aCINs are electrically coupled to other unidentified neurons, at least in the presence of 5-HT. First, we observed small rhythmic oscillations in aCINs during 5-HT application; the shape of these oscillations was suggestive of a truncated spike followed by a slower afterhyperpolarization (Figs. 5C and 6). Second, these oscillations were reversibly blocked by 100 \( \mu \text{M} \) carbenoxolone. Third, the oscillations were reversibly blocked by TTX, which would eliminate action potentials in the coupled neurons. Fourth, the amplitude of the oscillations did not vary with membrane potential of the aCIN, suggesting that it is not endogenously generated by the neurons themselves. Interestingly, these oscillations were seldom observed in the absence of 5-HT. It is possible that the neurons that are coupled to aCINs were silent at rest and only fire action potentials after applying 5-HT. The neurons providing these oscillations are not known. If they are also aCINs, this would allow the population of aCINs to provide a more coordinated pattern of input to the contralateral side of the spinal cord.

In summary, we have found that in the neonatal mouse spinal cord, the majority of aCINs fire rhythmically during fictive locomotion; most fire in phase with ipsilateral motoneuron activity. 5-HT, which plays a critical role in enabling the locomotor CPG, excites the aCINs by depolarizing their membrane potentials, reducing the voltage threshold for spiking, and reducing the amplitude of the AHP. aCINs are electrically coupled to unidentified neurons that become active primarily during fictive locomotion or serotonin application. These results support the hypothesis that aCINs play important roles in coordinating left–right movements during fictive locomotion, and thus may be component neurons in the locomotor CPG in neonatal mice.

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