Serotonin Modulates Dendritic Calcium Influx in Commissural Interneurons in the Mouse Spinal Locomotor Network

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Díaz-Ríos M, Dombeck DA, Webb WW, Harris-Warrick RM. Serotonin modulates dendritic calcium influx in commissural interneurons in the mouse spinal locomotor network. J Neurophysiol 98: 2157–2167, 2007. First published June 20, 2007; doi:10.1152/jn.00430.2007. Commissural interneurons (CINs) help to coordinate left–right alternating bursting activity during fictive locomotion in the neonatal mouse spinal cord. Serotonin (5-HT) plays an active role in the induction of fictive locomotion in the isolated spinal cord, but the cellular targets and mechanisms of its actions are relatively unknown. We investigated the possible role of serotonin in modifying dendritic calcium currents, using a combination of two-photon microscopy and patch-clamp recordings, in identified CINs in the upper lumbar region. Dendritic calcium responses to applied somatic voltage-clamp steps were measured using fluorescent calcium indicator imaging. Serotonin evoked significant reductions in voltage-dependent dendritic calcium influx in about 40% of the dendritic sites studied, with no detectable effect in the remaining sites. We also detected differential effects of serotonin in different dendritic sites of the same neuron; serotonin could decrease voltage-sensitive calcium influx at one site, with no effect at a nearby site. Voltage-clamp studies confirmed that serotonin reduces the voltage-dependent calcium current in CINs. Current-clamp experiments showed that the serotonin-evoked decreases in dendritic calcium influx were coupled with increases in neuronal excitability; we discuss possible mechanisms by which these two seemingly opposing effects can be reconciled. This research demonstrates that dendritic calcium currents are targets of serotonin modulation in a group of spinal interneurons that are components of the mouse locomotor network.

INTRODUCTION

Voltage-dependent calcium currents play important roles in the control of firing properties of neurons and their synaptic interactions in many invertebrate (Harris-Warrick 2002; Kits and Mansvelder 1996) and vertebrate motor systems (Catterall 1987; Taylor et al. 1983; Sabatini et al. 2001; Sah and Faber 2002). The flow of Ca$^{2+}$ entering neurons through voltage-activated channels is a prime target for many neuromodulators (Anwyl 1991; Cooke 2002; Elmslie 2003; Gerschenfeld et al. 1989). This modulation of Ca$^{2+}$ currents has significant effects on the intrinsic and synaptic properties of neurons involved in rhythmic behaviors such as feeding (Jacklet et al. 2006; Jo et al. 2005; Vehovszky et al. 2004), respiration (Fan et al. 2000; Lieske and Ramirez 2006; Muronov and Richter 1998), and locomotion (Carlin et al. 2000; Krüger et al. 2000; Laurienti and Blankenship 1997; Matsu-shima et al. 1993).

Serotonin (5-HT) is an essential neuromodulator in the generation of locomotor-like behaviors (termed “fictive locomotion”) in the isolated spinal cord of rodents (Gordon and Whelan 2006; Liu and Jordan 2005; MacLean et al. 1998; Madriaga et al. 2004; Ornstein et al. 2005). However, the cellular targets of serotonin action in the central pattern generator (CPG) network that organizes locomotion are poorly understood. We have begun to study serotonin’s effects on an identified population of spinal interneurons, the commissural interneurons (CINs; Butt and Kiehn 2003; Kiehn and Butt 2003; Lanuza et al. 2004; Zhong et al. 2006a,b). The CINs send their axons across the midline in the spinal cord and have essential roles in the organization of left–right coordination during locomotion (Butt and Kiehn 2003; Butt et al. 2002; Lanuza et al. 2004). The potential role of the CINs as key members in the generation and/or induction of the locomotor pattern remains unsolved (see Kiehn 2006). We and others have recently shown that 5-HT increases the excitability of the ascending (aCINs; Carlin et al. 2006; Zhong et al. 2006a) and descending (dCINs; Zhong et al. 2006b) CINs, whose axons ascend or descend, respectively, after crossing the midline (Eide et al. 1999; Nissen et al. 2005). 5-HT increased the excitatory responsiveness of aCINs and dCINs by depolarizing the membrane potential, reducing the postspike afterhyperpolarization amplitude, and decreasing the action potential threshold (Zhong et al. 2006a,b). The ionic mechanisms underlying these effects are unknown.

Here, we report the results of combined patch-clamp and multiphoton microscopy (Denk and Svoboda 1997; Denk et al. 1990, 1996) calcium imaging experiments to analyze changes in voltage-evoked Ca$^{2+}$ influx in CIN dendrites. We previously used these methods to determine the location and dopamine (DA) modulation of neurite calcium currents in neurons of a simple invertebrate CPG, the pyloric network in the lobster stomatogastric ganglion (STG; Kloppeburg et al. 2000). That research showed that Ca$^{2+}$ accumulation originates mostly from small spatially restricted varicosities on distal neurites and that Ca$^{2+}$ accumulation is primarily decreased by DA in pyloric dilator (PD) neurons. This correlated well with dopamine’s inhibition of the PD neurons (Flamm and Harris-Warrick 1986) and reduction of PD synaptic outputs (Johnson and Harris-Warrick 1990).

Based on the excitatory effects of 5-HT on two types of CINs (Carlin et al. 2005; Zhong et al. 2006a,b) we expected to see 5-HT–induced increases in Ca$^{2+}$ accumulation in CINs. Instead, we found that serotonin either reduces or has no effect
on voltage-activated calcium influx in dendrites of CINs. This result was confirmed by voltage-clamp studies, showing that the voltage-activated calcium currents measured in CIN somata were also significantly reduced by serotonin. Additional current-clamp experiments suggest that a calcium-activated potassium current \( [K_{Ca}] \) might be a target of serotonin modulation. The possible relation between these results and the excitatory effects of 5-HT on CINs is discussed.

**METHODS**

**Slice preparations**

Experiments were performed using spinal cords of 0- to 5-day-old (P0–P5) ICR mice (Taconic Farms, Hudson, NY). The animal protocol was approved by the Cornell University Institutional Animal Care and Use Committee and was in accordance with National Institutes of Health guidelines. Animals were killed by rapid decapitation. The spinal cord was 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 C5 to S3 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.

CINs were fluorescently labeled retrogradely by making fine slits in the contralateral hemicord rostrally (T13–L1) and caudally (L4 –L5) of the target L2 region, and applying crystals of fluorescent dextran (e.g., Texas Red or Alexafluor). Preparations were incubated in oxygenated mouse Ringer solution at 30°C for 1–2 h to allow the dyes to 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 solution.

**Drugs**

The glutamate
gic antagonists d-(-)-2-amino-5-phosphonopentanoic acid (AP-5), and 6-cyano-7-nitroquininaline-2,3-dione disodium salt (CNQX), and the small-conductance Ca2+-dependent K+ (SK) channel blocker apamin were purchased from Tocris (Ellisville, MO). The γ-aminobutyric acid (GABA) antagonist picrotoxin, the glycine antagonist strychnine, the transient potassium current antagonist 4-aminopyridine (4-AP), potassium channel antagonist tetraethylammonium (TEA), and sodium channel antagonist tetrodotoxin (TTX) were purchased from Sigma (St. Louis, MO).

**Calcium Green-1 dye filling and recording**

Whole cell patch-clamp recordings were obtained under infrared video microscopy using 5- to 10-MΩ pipettes pulled from thick-wall borosilicate glass (Sutter Instrument, Novato, CA) filled with intracellular solution (in mM): 138 K-glucuronate, 10 HEPES, 0.0001 CaCl2, 5 Mg-ATP, 0.3 Li-GTP (pH 7.3 with KOH, osmolarity ≈ 260 mosmol/l). A final concentration of 200 μM Calcium Green-1 Dextran (3,000 MW) was added to this solution and then passed through a 0.2-μm filter. Unless the seal resistance before breakthrough was >1 GΩ, the neuron was discarded. Patch-clamp recordings were made with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and were driven by Clamp programs (pClamp 8.1, Axon Instruments). Under our experimental conditions, the electrode’s liquid junction potential was about 8 mV (with the cell more hyperpo-

**FIG. 1.** Measurement of voltage-induced calcium transients in commissural interneurons (CINs). A: schematic drawing of the labeling method for CINs in spinal cord slices. CINs were labeled with Texas Red dextran amine (red) applied to contralateral slits between roots T13–L1 and L3–L4. Transverse slices were prepared and CINs located primarily within lamina VII were identified and patch clamped under epifluorescence. B: Z-projection image of a CIN patch clamped and filled with Calcium Green-1 dextran. Note the bright fluorescent areas, which we refer to as bright dendritic regions (arrowheads). C: a single optical section (thickness: −1–2 μm) of the CIN in B with the line scan position marked with a white line. Branches that are only partly or not present in the optical section are either dim or not visible. D: top: line scan recording from position in C. A voltage step to about 0 mV (100 ms) was given to the soma at the time noted (asterisk). Note that the fluorescence increases in the dendritic spots during this step. Bottom: a fluorescence vs. time plot (extracted from the top line scan trace) at the dendritic spot indicated in C. Temperal resolution was 2 ms per line. Timescale is the same as that in D (top). E: plot of ΔF/F vs. time for 20 min with 1 (filled squares) or 5 (open circles) min between line scans using our original microscope setup and at 90 s between line scans (filled circles) after increasing the photon collection efficiency of our microscope, decreasing the number of collected lines, and adding a Pockel’s cell to reduce the integrated amount of illumination.

**Two-photon microscopy**

The basic design of our Radiance 2000 (Bio-Rad)–based imaging system has been previously described (Dombeck et al. 2003, 2004). Briefly, an upright microscope (Olympus, BX50WI) was used with a
Calcium imaging of CINs

After about 10–15 min of whole cell recordings, the impaled cell was usually well labeled with Calcium Green-1 dextran including the soma and proximal dendrites, and some distal dendrites (Fig. 1B). As subsequently described, the dye distributed unevenly in the dendrites, with brighter regions corresponding to high concentrations of dye; we focused on these for our recordings because they gave higher signal-to-noise (S/N) recordings. CINs were isolated from most rapid synapses, and strychnine (10 mM) to block glycinergic synapses, and picrotoxin (10 mM) to block GABAergic synapses as previously described. Some of the neurons fired spontaneously (n = 8), whereas others were silent at rest (n = 11; Vm = –55.5 ± 4.8). To ensure uniformity of the measurements, all neurons were held below threshold at –68 mV with a bias current. Only neurons with action potentials whose peak amplitudes were >0 mV were included in the analysis. The 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, with respect to ground. Action potentials for AHP measurements were elicited by brief high-current pulses (5–10 ms/1 nA) applied at holding potential (~68 mV). Input resistance was measured by applying small constant hyperpolarizing voltage pulses (5 mV) in the voltage-clamp mode. To generate the frequency–current (F–I) plot, a series of increasing amplitude current steps (1 s) were given and the initial instantaneous frequency during the step was determined by calculating the inverse of the time duration between the first two spikes. The effects of serotonin on the membrane properties were analyzed by paired two-tailed Student’s t-test. Results were considered statistically significant at P < 0.05. Data are expressed as means ± SD.

Optimization of calcium signal measurement with minimal photodamage

At least four measurements were needed under each condition (control, 5-HT, and washout) to generate enough data for adequate tests of statistical significance. We have found that the effects of 5-HT on CINs are poorly reversible if the 5-HT is left on the preparation for >5 min (Díaz-Ríos et al., unpublished observations). This requires line scanning approximately every minute to collect the desired number of time points. During our initial studies, we used only one epi-collecting BiAlkali PMT and no Pockel’s cell (voltage-controlled wave plate) for fly-back laser blanking; it was necessary to average two line scans at every time point to increase the S/N for adequate tests of significance. After 20 min of recordings at 1-min intervals, we found that voltage-induced Ca2+ influx at a specific dendritic site was reduced to 38 ± 6% of its original value (Fig. 1E), which we attributed to photodamage. If we reduced the line scan frequency to every 5 min instead of every minute, the photodamage was significantly reduced, and the signal was 90 ± 38% of the original value after 20 min (Fig. 1E). However, obtaining four line scans during 5-HT application would take >20 min, much longer than desired to maintain a healthy preparation.

We therefore modified our imaging setup to reduce photodamage. We installed a PMT in the transmitted light direction to nearly double the amount of collected light. We replaced the BiAlkali PMTs with GaAsP PMTs, which doubled the collection efficiency. The number of lines per scan was reduced from 1,000 to 400 (still enough to measure the peak change in fluorescence due to calcium influx). Finally, a Pockel’s cell (Conoptics, Danbury, CT) was added for laser fly-back blanking, reducing the total laser illumination by about 50%. With these improvements, it was possible to obtain the same or better S/N ratio without averaging at every time point. We repeated the photodamage studies and found that it was possible to take a line scan about every 90 s and still have the Ca2+ response at 89 ± 25% of the original value after 20 min (Fig. 1E). These considerations set our imaging parameters and experiment timescale. This required 5-HT to remain in the bath for about 6–7 min, which compromised the reversibility of our drug applications but kept photodamage to a minimum.

Custom-made programs were used to analyze dendritic calcium dynamics (LabVIEW 6.1 with Vision Development Module 6.1). Plots and graphs were prepared using Origin 7 and Sigma Plot 2001. Figures and brightness/contrast adjustments were made using Adobe Photoshop 6.1 and Corel Draw 9.

Firing properties of isolated CINs

The CINs were isolated from most rapid synaptic inputs with a combination of blockers for glutamatergic (AP-5 and CNQX), GABAergic (picrotoxin), and glycinergic (strychnine) synapses as previously described. Some of the neurons fired spontaneously (n = 8), whereas others were silent at rest (n = 11; Vm = –55.5 ± 4.8). To ensure uniformity of the measurements, all neurons were held below threshold at –68 mV with a bias current. Only neurons with action potentials whose peak amplitudes were >0 mV were included in the analysis. The 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, with respect to ground. Action potentials for AHP measurements were elicited by brief high-current pulses (5–10 ms/1 nA) applied at holding potential (~68 mV). Input resistance was measured by applying small constant hyperpolarizing voltage pulses (5 mV) in the voltage-clamp mode. To generate the frequency–current (F–I) plot, a series of increasing amplitude current steps (1 s) were given and the initial instantaneous frequency during the step was determined by calculating the inverse of the time duration between the first two spikes. The effects of serotonin on the membrane properties were analyzed by paired two-tailed Student’s t-test. Results were considered statistically significant at P < 0.05. Data are expressed as means ± SD.

Voltage-clamp analysis of I_{Ba}

To study the effects of 5-HT on voltage-activated Ca2+ currents (I_{Ca}), synaptically isolated CINs were voltage clamped with a Multiclamp 700A amplifier (Axon Instruments) driven by Clampex programs (pClamp 9, Axon Instruments) in the presence of blockers for sodium (TTX, 1 μM), potassium (TEA, 10 mM; 4-AP, 4 mM), and calcium-activated potassium currents (I_{KCa}; apamin, 100 nM). Additionally, extracellular Ca2+ was replaced with Ba2+, which enhances the current through calcium channels and further reduces potassium and calcium-activated currents (Mayer 1985; Veselovskii and Fedulova 1986). Voltage steps in 10-mV increments between –50 and +30 mV were delivered to activate I_{Ba}. For these voltage-clamp studies the liquid junction potential was corrected on-line to the time of each experiment. After each experiment, cadmium (CdCl2; 500 μM) was added to the perfusate to block all calcium currents and the steps were repeated; these were digitally subtracted from control, 5-HT, and wash voltage traces to eliminate any residual outward currents. Linear leakage and capacitative currents were digitally subtracted with a P/6 protocol (see Bezanilla and Armstrong 1974). Series resistance and capacitance were compensated to ≥70% in every experiment.

RESULTS

Voltage-induced Ca2+ influx measurements in CINs

CINs were identified by retrograde fluorescent labeling with Texas Red dextran amine placed in fine slits in the contralateral
hemicord rostrally (T13–L1) and caudally (L4–L5) of the target L2 region as previously described (Fig. 1A; see Glover 1995); we did not distinguish between ascending, descending, or bifurcating CINs. We selected Calcium Green-1 as the Ca\(^{2+}\) indicator dye for the CINs because it loads quickly into cells, provides adequate fluorescence intensity, and shows no significant leakage out of the cell over time. In our previous research, Calcium Green-1 produced high S/N responses to a single voltage step in STG neurons (Kloppenburg et al. 2000). Calcium Green-1 worked well for dye loading CINs (see Fig. 1, B and C) that, after establishing the whole cell configuration with the patch pipette, were consistently filled within 15 min (Fig. 1B). After this time, we were routinely able to visualize primary, secondary, and higher-order dendrites up to distances of approximately 75 microns from the soma. Excellent three-dimensional images with high spatial resolution could be obtained ≤100 μm below the surface of the slice. At the end of each experiment, a full z-series of each investigated neuron was collected to reconstruct the cell’s morphological features (Fig. 1B).

After CINs were filled with Calcium Green, we consistently observed that within the filled dendrites of these neurons there were regions a few microns in diameter that presented a higher fluorescence signal than that of the surrounding area (Fig. 1B, arrowheads). Using a ratiometric dye (Indo-1) we found that the calcium concentration ([Ca\(^{2+}\)]) throughout the soma and dendritic arbor was the same (P = 0.94 for statistical comparison between brighter and dimmer regions of the arbor; P = 0.10 for statistical comparison between soma and brighter regions and between soma and dimmer regions of the arbor). Thus any brighter regions seen with Calcium Green were a result of higher dye accumulation as opposed to a higher [Ca\(^{2+}\)]. Because of the increased fluorescence signal provided by regions with higher dye accumulation, we focused primarily on these spots for testing dendritic Ca\(^{2+}\) influx sensitivity to applied serotonin (5-HT, 9 μM). We also used Indo-1 to determine the average resting calcium level in the CINs, which was 200 ± 150 nM (n = 4).

After establishing the recording, CINs were superfused with Ringer solution containing the synaptic blockers AP-5 (15–20 μM) and CNQX (30 μM) to block glutamatergic synapses, picrotoxin (10 μM) to block GABAergic synapses, and strychnine (10 μM) to block glycinergic synapses. The combination of these effectively eliminated all detectable rapid synaptic inputs to the neurons, although it would not remove slower modulatory inputs. To evoke Ca\(^{2+}\) influx, the soma was voltage clamped from a holding potential of −68 to −8 mV for 100 ms. An example of the methodology used for the acquisition and analysis of these voltage-induced Ca\(^{2+}\) signals is shown in Fig. 1, C and D. We used the line scan mode in which single lines crossing specific regions of dendrites (Fig. 1C, white line) were scanned in succession at intervals of 2 ms to maximize temporal resolution. Line scan images were constructed by sequentially displaying these line scans in a column that is shown horizontally in Fig. 1D (top) for better alignment with respect to time with the bottom panel. Changes in fluorescence intensity over time were extracted from regions of the line scan images and expressed as the change of this signal normalized to the basal level of fluorescence intensity (ΔF/ΔF\(_{0}\); Fig. 1D, bottom). As seen in Fig. 1D (bottom), the fluorescent signal displayed a rapid rise during the applied voltage step and a slower decay, lasting up to a few seconds, after termination of the step.

**Voltage dependence of the induced Ca\(^{2+}\) signal**

Based on the relatively close proximity of our recording sites in the dendritic arbor to the soma (32 ± 14-μm distance, ranging from 7 to 64 μm), we expected to achieve reasonable voltage control of these sites when voltage clamped from the soma. We obtained a consistent and reproducible measure of the voltage dependence of the fluorescent Ca\(^{2+}\) accumulation signal. This is shown in Fig. 2 where 100-ms voltage steps were applied to the soma of CINs from a holding potential of −68 mV. Ca\(^{2+}\) signals were detected during voltage steps more depolarized than −40 mV, with a peak at about +20 mV (Fig. 2, A and B). This peak value occurs at a somewhat more

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**FIG. 2.** Voltage and calcium dependence of the induced Ca\(^{2+}\) influx in a CIN. A: *three traces* show the kinetics of the influx and decay with 3 different voltage pulse amplitudes marked on the plots (colors correspond to the membrane potential reached during the voltage step). Holding potential is −68 mV. *Black trace* (cell kept at original holding potential; no change in voltage) is also shown to demonstrate that photobleaching of the Ca\(^{2+}\) indicator dye did not occur during line scan acquisition. B: *maximum Ca\(^{2+}\)* signal as a function of the voltage pulse amplitude. Voltage pulses (100 ms) of varying amplitude were applied to the soma, and the Ca\(^{2+}\) influx was monitored in active dendritic regions in the neuropil. Pooled data from 3 different CINs are shown. C: *plot of ΔF/ΔF* vs. time showing the Ca\(^{2+}\) signal of a CIN before, during, and after replacing the regular extracellular Ringer solution with a 10% [Ca\(^{2+}\)] Ringer. Notice the reversible reduction in the voltage-activated Ca\(^{2+}\) influx. D: *pooled data* showing a statistically significant reversible reduction of the Ca\(^{2+}\) signal after perfusing the 10% [Ca\(^{2+}\)] extracellular Ringer (P < 0.01; n = 3). E: extracellular application of 500 μM CdCl\(_{2}\) completely abolished the Ca\(^{2+}\) signal in a nonreversible manner. F: *pooled data* showing the abolishment of the Ca\(^{2+}\) signal in the CINs after CdCl\(_{2}\) application (P < 0.01; n = 3).
depolarized membrane potential than we would expect from voltage-clamp studies. We attribute the difference to the imperfect ion selectivity of calcium channels for K⁺ and Na⁺, which hyperpolarize its apparent equilibrium potential from $E_{Ca}$ when measured by voltage clamp; in contrast, the imaging measurements accurately reflect calcium entry alone. These results provide evidence for a good voltage dependence of the Ca²⁺ signal of the CINs, suggesting that they are mediated by voltage-activated channels.

As expected, the voltage-induced Ca²⁺ influx was dependent on extracellular Ca²⁺. When the extracellular solution was replaced with a low calcium Ringer solution (10% of the normal concentration), the Ca²⁺ signal was significantly and reversibly reduced to 62.0 ± 9.3% of the value in normal solution (Fig. 2, C and D; normal vs. low Ca²⁺: $P < 0.001$; low Ca²⁺ vs. wash: $P = 0.001$; $n = 3$). This reduction is of the correct order of magnitude if the Ca²⁺ driving force, Calcium Green fluorescence versus Ca²⁺ concentration relationship, and dye concentrations are all taken into account. Finally, the Ca²⁺ response was completely abolished when calcium currents were blocked by the addition of 500 µM cadmium to the superfusate (Fig. 2, E and F; $n = 3$), providing further evidence that the voltage-activated signal is mediated by voltage-activated Ca²⁺ channels.

Serotonin modulation of Ca²⁺ signals in CINs

To investigate the effects of 5-HT on voltage-dependent dendritic Ca²⁺ influx, line scans were recorded from identified dendritic sites of voltage-dependent calcium influx. These signals were tested for stability over a period of 5 min by 100-ms voltage steps from −68 to −8 mV. Voltage steps were applied every 90 s, which minimizes photodamage from repeated irradiation of the site (see METHODS and Fig. 1E). Only sites with consistent responses over this time were used for studying the effects of bath-applied 5-HT (9 µM).

The effects of 5-HT on the voltage-evoked Ca²⁺ signal were quantified by comparing the peak amplitude, rise time, and decay time of the signal after the voltage step in control conditions and during bath application of 9 µM 5-HT. The average of four different line scans under each experimental condition (control, 5-HT, wash) was expressed as the change of signal normalized to the basal level of fluorescence intensity ($\Delta F/F$), as described earlier. In 40% of the dendritic spots studied, 5-HT produced a significant decrease in the recorded Ca²⁺ signal (13/33 dendritic spots; 8/15 cells; $P < 0.05$; examples in Fig. 3, A and B, red traces and bars). As we found previously with prolonged applications of 5-HT to CINs, its effects reversed poorly over the time of our recordings (5- to 10-min washout). Thus to confirm that the observed decrease of Ca²⁺ influx was an effect of 5-HT modulation, and not simply photodamage induced by the line scanning procedure or washout of intracellular signaling components, we performed control experiments with the same protocol and duration of recording, but without 5-HT application. An example of this control experiment is shown in Fig. 3A (black traces), where recordings from two different dendritic regions are shown. After the entire recording period (~20 min), the calcium signal decreased significantly in only one of the scanned dendritic spots (32 total dendritic spots, $n = 8$), which is expected with our set $P$ value of 0.05. Comparison of pooled data from these 32 control dendritic spots with the 13 spots with reduced Ca²⁺ signals during 5-HT application confirms that the observed decreases in Ca²⁺ influx were produced by 5-HT (Fig. 3B; control, black bar; 5-HT, red bar; $P < 0.001$). The average decrease in Ca²⁺ signal due to serotonin was calculated by comparison with the average signal in spots monitored for the same time but not exposed to 5-HT, and was 25 ± 13%. In 2 of the 13 spots (1/15 cells), there was a significant recovery of the calcium signal (washout) after 5-HT ($P < 0.05$), but in the remaining spots and, on average, the response reversed poorly.
shown (Zhong et al. 2006a,b), application of 9-mined the amplitude of current steps that evoked 10-Hz firing To further quantitate the increase in excitability, we deter-

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Ca²⁺ significant change). Finally, we measured voltage-evoked 

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soma. These findings demonstrate that the effects of 5-HT are 

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Ca²⁺ influx during 5-HT application in dendrites, but not in the 

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CINs in which 5-HT reduced calcium influx in a dendritic site 

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CINs (seven total dendritic and somatic pairs; two recordings from 

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different dendritic and somatic sites were performed in two 

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CINs). We found that three of seven paired recordings showed 

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a significant decrease in calcium influx during 5-HT applica-

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CINs in which 5-HT reduced calcium influx in a dendritic site 

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CINs). We determined that three of seven paired recordings showed 

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a significant decrease in calcium influx during 5-HT application in dendrites and soma. In the remaining recordings 

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we found a significant decrease in calcium influx during 5-HT application in dendrites, but not in the soma. These findings demonstrate that the effects of 5-HT are not uniform in the dendrites and/or soma of single CINs.

Serotonin modulation of firing properties of recorded CINs

During the majority of the 5-HT experiments (9/15 cells), we also measured the effects of 5-HT on the excitability and input resistance of the CINs after blockade of glutamatergic, GABAergic, and glycineric transmission. First, we injected current steps of increasing amplitude and generated F–I plots of the instantaneous firing frequency over a range of current-step amplitudes. 5-HT caused an upward shift in the instantaneous frequency response to the current steps and evoked a significant increase in the slope of the F–I relationship in 75% of the CINs (Fig. 3D; control: 0.37 ± 0.19 Hz/pA; 5-HT: 0.5 ± 0.27 Hz/pA; wash: 0.46 ± 0.25 Hz/pA; n = 6/8, P = 0.014). To further quantitate the increase in excitability, we determined the amplitude of current steps that evoked 10-Hz firing responses under control conditions. As we have previously shown (Zhong et al. 2006a,b), application of 9 μM 5-HT significantly increased the firing frequency of the majority of the CINs to this step (control: 10.2 ± 1.1 Hz; 5-HT: 13.6 ± 4.2 Hz; washout: 12.4 ± 2.8 Hz; n = 9/11; P < 0.05; Fig. 3C). We additionally monitored the cell input resistance by measuring the current response to small hyperpolarizing (5-mV) voltage pulses under voltage clamp. In accordance with our previous results (Zhong et al. 2006a,b), we found that the input resis-
tance was not significantly increased after the application of 5-HT (control: 452 ± 219 MΩ; 5-HT: 460 ± 272; wash: 455 ± 247; n = 9; P = 0.25). These results suggest that the 5-HT-evoked reduction in Ca\(^{2+}\) influx was due to a modulatory effect of 5-HT and not simply a result of progressive loss of our voltage clamp due to a reduction in input resistance over time (Klopfenborg et al. 2000).

Voltage-clamp analysis of calcium currents

The imaging data indicates that voltage-activated calcium currents (I\(_{Ca}\)) are modulated in CINs by 5-HT. To confirm this result, voltage-clamp measurements of calcium currents were performed on CINs. To isolate these currents, we added TEA (10 mM), 4-AP (4 mM; K\(^+\) channel blockers), and TTX (1 μM; Na\(^+\) channel blocker) and replaced all extracellular Ca\(^{2+}\) (∼2 mM) with elevated levels of Ba\(^{2+}\) (10 mM), which enhances the current amplitude through calcium channels and also reduces potassium and calcium-activated potassium conductances. CINs were held at −50 mV and given 200-ms depolarizing voltage steps in 10-mV increments to +30 mV (Fig. 5A). Under control conditions, the leak-subtracted I\(_{Ba}\) activated during voltage steps above −40 mV (Fig. 5, A and B); as expected, the current reaches a maximum near 0 mV and then falls with the decreasing driving force on the ion. I\(_{Ba}\) was completely eliminated by 500 μM CdCl\(_2\), confirming that these inward currents were through Ca\(^{2+}\) channels (Fig. 5B). 5-HT (9 μM) significantly reduced the voltage-activated Ba\(^{2+}\) current (Fig. 5A; n = 9). Current–voltage (I–V) curves for activation of I\(_{Ba}\) were made under control, 5-HT, and wash conditions (Fig. 5B). 5-HT significantly decreased the conductance of the Ba\(^{2+}\) current in the majority of the CINs tested by 49 ± 20% (n = 7/9 CINs, P < 0.05) and, in some experiments, this effect was partially reversible (Fig. 5B). There was no effect of serotonin on the voltage dependence of the current or on the activation or inactivation kinetics of I\(_{Ba}\) in the CINs (Fig. 5A; data not shown). These results further confirm that 5-HT inhibits voltage-activated Ca\(^{2+}\) currents. The voltage-clamp experiments also demonstrate that the effects of 5-HT, monitored in specific dendritic spots through our imaging experiments, are also detectable in the soma.

Current-clamp analysis of the effect of the SK-channel blocker apamin on the intrinsic properties of the CINs

Serotonin’s reduction of the Ba\(^{2+}\) current (seen in the voltage-clamp studies) and of Ca\(^{2+}\) influx (observed in our imaging studies), coupled with the excitatory effects of 5-HT on the intrinsic properties of the CINs (Zhong et al. 2006a,b), prompted us to examine whether 5-HT could indirectly inhibit a calcium-activated potassium current \([I_{K(Ca)}]\) as a consequence of the reduction in I\(_{Ca}\). We explored this possibility by studying the effects of the SK-channel blocker apamin on the action potential afterhyperpolarization (AHP) and firing properties of the CINs. Apamin (100 nM) significantly decreased the AHP in all of the CINs studied (Fig. 6; control: 5.6 ± 2.1 mV; apamin: 1.5 ± 1.8 mV; n = 10, P < 0.01). In support of our hypothesis, subsequent application of 5-HT had no additional effects on the AHP amplitude in the presence of apamin (Fig. 6; apamin + 5-HT: 1.4 ± 1.8 mV, P = 0.75). Apamin also mimics and occludes 5-HT’s increase of CIN excitability (Fig. 7), measured as the F–I relationships (Fig. 7B). Like 5-HT, apamin increased the instantaneous firing frequency at each step (Fig. 7B), as seen by an upward shift in the F–I curve. Additional 5-HT application had no significant effects on the F–I relationship of the CINs (Fig. 7B) and did not further increase the instantaneous firing frequency of the CINs in response to a current step that evoked a 10-Hz response under control conditions (Fig. 7C; control: 9.9 ± 1.2 Hz; apamin: 27.4 ± 16.7 Hz; apamin + 5-HT: 29.8 ± 17.3 Hz; n = 10, P =

![Fig. 5](http://jn.physiology.org/) 5-HT modulation of voltage-activated Ca\(^{2+}\) currents in the commissural interneurons (CINs). A: current traces from a CIN in the presence of tetraethylammonium (TEA, 10 mM) and 4-aminoypyridine (4-AP, 4 mM), tetrodotoxin (TTX, 1 μM), and with all extracellular Ca\(^{2+}\) replaced with Ba\(^{2+}\), before, during, and after 5-HT (9 μM) application. B: current–voltage (I–V) curve for activation of I\(_{Ba}\) during control, 5-HT, wash, and after the application of Cd\(^{2+}\) (500 μM) to the Ringer.
0.34). These results suggest that 5-HT’s reduction of $I_{Cn}$ leads indirectly to a reduction of $I_{K(Ca)}$, and that this action may play an important role in 5-HT–evoked increases in CIN excitability.

**DISCUSSION**

Serotonin plays an important role in the induction of fictive locomotion in the isolated rodent spinal cord (Christie and Whelan 2005; Liu and Jordan 2005; MacLean et al. 1998; Madriaga et al. 2004; Pearlstein et al. 2005). Here we studied the effects of 5-HT on voltage-induced Ca$^{2+}$ influx in commissural interneurons (CINs). Previous research on the CINs has provided evidence in support of their role as component neurons within the rodent spinal locomotor network (Butt and Kiehn 2003; Lanuza et al. 2004; Nishimaru et al. 2006; Quinlan and Kiehn 2005; Zhong et al. 2006a,b). Given the importance of the CINs in coordinating left–right alternating locomotor activity (Lanuza et al. 2004), we sought to understand how their intrinsic firing properties change under conditions that evoke fictive locomotion in the intact cord. Using multiphoton microscopy, we studied whether 5-HT modulates voltage-dependent calcium influx in CIN dendrites. Spinal motorneurons are known to have amine-enhanced persistent inward currents that arise from a combination of sodium- and calcium-dependent currents (Heckman et al. 2005; Lee et al. 1999; Powers and Binder 2003; Zeng et al. 2005). We used multiphoton microscopy to image the voltage-dependent calcium currents in CIN dendrites to determine whether calcium currents could be affected by serotonin in these cells.

In Calcium Green–labeled CINs, specific dendritic sites showed higher resting fluorescent signals than the surrounding areas (Fig. 1). However, using the ratiometric dye Indo-1 we found that these areas resulted from accumulation of dye and not local regions of higher [Ca$^{2+}$]. Ca$^{2+}$ entry is not strictly spatially localized to specific compartments within the dendrites of the CINs, as was found earlier in the lobster STG (Kloppenburg et al. 2000), suggesting that voltage-gated calcium channels are more evenly distributed throughout the dendritic arbor.

The observed Ca$^{2+}$ influx after applied voltage steps showed a reproducible and consistent voltage dependence; these signals were reduced by low-Ca$^{2+}$ solutions and blocked by Cd$^{2+}$-containing solutions, respectively, demonstrating that voltage-dependent calcium influx in CIN dendrites. Spinal motorneurons are known to have amine-enhanced persistent inward currents that arise from a combination of sodium- and calcium-dependent currents (Heckman et al. 2005; Lee et al. 1999; Powers and Binder 2003; Zeng et al. 2005). We used multiphoton microscopy to image the voltage-dependent calcium currents in CIN dendrites to determine whether calcium currents could be affected by serotonin in these cells.

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activated Ca\textsuperscript{2+} channels were the sites of Ca\textsuperscript{2+} entry. Additionally, Ca\textsuperscript{2+}-activated Ca\textsuperscript{2+} release from intracellular stores could contribute to the observed Ca\textsuperscript{2+} signals (Kloppenburg et al. 2000). However, based on previous studies by our laboratory looking at the modulation of Ca\textsuperscript{2+} influx by dopamine in lobster neurons (Kloppenburg et al. 2000), if release of calcium from intracellular stores and reduction in calcium driving force constituted the principal reason for the observed 5-HT--induced decreases in calcium signal, we would have expected a significant increase in the baseline calcium signal (at the holding potential, before the voltage steps), which was not observed. The use of intracellular Ca\textsuperscript{2+} chelators such as BAPTA should reveal the actual contribution of intracellular Ca\textsuperscript{2+} stores to the Ca\textsuperscript{2+} signal. Characterization of the specific types of Ca\textsuperscript{2+} channels being modulated by 5-HT in the CINs will be performed in future experiments.

In this study we were particularly interested in the effects of 5-HT on voltage-induced Ca\textsuperscript{2+} influx in the dendrites of the CINs. Despite the excitatory effects of 5-HT on CINs, we found that 5-HT (9 μM) significantly reduces Ca\textsuperscript{2+} influx in 40% of the investigated dendritic spots, while having no effect at virtually all of the remaining spots. This effect did not readily reverse; we found that there is a 5-min “time window” for serotonin application, after which the physiological effects of 5-HT reverse poorly. Because of the limitations for data acquisition without photodamage (see METHODS), it was necessary to apply 5-HT for ≈6–7 min, which may explain its poor reversibility. This long-term effect of 5-HT could be due in part to activation of additional signaling pathways. Machacek et al. (2001) also observed long-term excitatory effects of 5-HT, by 5-HT\textsubscript{2} receptors, on deep dorsal horn neurons; these effects lasted long after 5-HT washout. Control experiments monitoring dendritic voltage-dependent calcium influx without the addition of 5-HT did not show a signal decrease (Fig. 3, A and B), confirming that reduction of voltage-dependent Ca\textsuperscript{2+} influx is a 5-HT--induced effect. To further verify these results, we used voltage-clamp experiments to demonstrate that 5-HT also significantly reduces voltage-activated Ca\textsuperscript{2+} currents measured from the soma of CINs. These experiments did show at least partial reversibility, which was probably due to the shorter application time for 5-HT in these experiments.

Given that serotonin excites both aCINs and dCINs (Zhong et al. 2006a,b), we were surprised that 5-HT did not increase their voltage-induced Ca\textsuperscript{2+} influx. Berger and Takahashi (1990) showed that serotonin enhances a low-voltage--activated Ca\textsuperscript{2+} current in rat spinal motor neurons and that this effect, together with a previously reported 5-HT--induced inward rectifying current (Takahashi and Berger 1990), increases their excitability. On the other hand, Bayliss et al. (1995) and Ladewig et al. (2004) found that 5-HT, acting through 5-HT\textsubscript{1} receptors, decreased calcium currents in hypoglossal motoneurons and excited the neurons by a decrease in the spike afterhyperpolarization (AHP). The significant decreases in calcium accumulation in 40% of the CIN dendritic spots probably do not reflect a 5-HT--induced effect on a specific type of CIN because it is known that the majority of CINs in the rodent spinal cord are either aCINs or dCINs (Eide et al. 1999; Stokke et al. 2002), and 80% of these neurons are excited by 5-HT (Carlin et al. 2006; Zhong et al. 2006a,b). In addition, this explanation is unlikely because we found that 5-HT can modulate Ca\textsuperscript{2+} influx differentially in locations of the same neuron, with one spot in the dendrite or soma showing a decrease in Ca\textsuperscript{2+} influx, whereas another spot in the same neuron showed no response to 5-HT (Fig. 4). A similar disparity of effect in a single cell was previously seen in the lobster STG (Kloppenburg et al. 2000). This differential effect of 5-HT in different regions of the same neuron could be explained by the differential expression of channel or receptor types at distinctive sites in the neuron. Previous research has shown that Ca\textsuperscript{2+} influx can occur at different dendritic locations by different pathways, such as voltage-sensitive Ca\textsuperscript{2+} channels, N-methyl-D-aspartate receptors, and Ca\textsuperscript{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or kainate receptors (Denk et al. 1995; Euler et al. 2002; Goldberg et al. 2003; Pellistri et al. 2004; Wang et al. 2002; Yuste et al. 1999). These could express different levels of sensitivity to 5-HT. Additional experiments and modeling studies will be necessary to explain the actual physiological significance of these results.

It is not yet clear how this reduction in I\textsubscript{Ca} relates to the excitatory effects of 5-HT on aCINs and dCINs (Zhong et al. 2006a,b). One possibility is that a reduction in I\textsubscript{Ca} indirectly inhibits a calcium-activated potassium conductance to enhance spiking, as demonstrated in rat hypoglossal motoneurons (Bayliss et al. 1995; Ladewig et al. 2004) and in lamprey motorneurons (Hill et al. 2003). We previously found that 5-HT reduces the amplitude of the postspike AHP in CINs, which appear to be mediated mainly by an SK-type I\textsubscript{K(Ca)} (Zhong et al. 2006a,b; Díaz-Ríos et al., unpublished results). Further supporting our hypothesis, we found that the SK-channel blocker apamin mimics and occludes serotonin’s effects on the AHP (Fig. 6). 5-HT has been shown to suppress the AHP and excite cholinergic interneurons of the rat striatum (Blomeley and Bracci 2005) and in layer V pyramidal neurons of the prefrontal cortex (Villalobos et al. 2005). I\textsubscript{K(Ca)} has been shown to mediate a majority of the AHP of rat motoneurons (Gao and Ziskind-Conhaim 1998) and lamprey spinal neurons (Wikstrom and El Manira 1998).

Decreases in I\textsubscript{K(Ca)} not only shifted the instantaneous frequency–current (F–I) relationship upward (more spikes per current step) but also increased its slope (Bond et al. 2004; Hallworth et al. 2003; Perez-Rosello et al. 2005; Sourdet et al. 2003). In our study, 5-HT and apamin increased both of these parameters and apamin occluded the effects of 5-HT (Fig. 7). In an earlier study (Zhong et al. 2006a,b), we reported a parallel increase in the F–I relationship after 5-HT application, with no obvious change in slope on the CINs; however, we were measuring the average spike frequency over the 1-s spike train instead of the instantaneous frequency at the beginning of the spike train in those studies. Although our research suggests that serotonin’s modulation of I\textsubscript{K(Ca)} due to a decrease in calcium entry is a major contributor in serotonin’s excitatory effects, additional currents may also be involved, such as the persistent sodium current (Carr et al. 2002; Tanaka and Chandler 2006) or low-threshold calcium currents. More studies are needed to further characterize the modulatory actions of 5-HT on specific channels and channel types in the spinal CINs.

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