Multiple Effects of Serotonin and Acetylcholine on Hyperpolarization-Activated Inward Current in Locomotor Activity-Related Neurons in Cfos-EGFP Mice

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Submitted 16 December 2009; accepted in final form 11 April 2010


Hyperpolarization-activated inward current (Ih) in the spinal cord has been shown to be involved in the production of bursting during various forms of rhythmic activity. However, the biophysical properties of Ih in spinal interneurons related to locomotion remain unknown. Using Cfos-EGFP transgenic mice (P6–P12) we were able to target the spinal interneurons activated by locomotion. Following a locomotor task, whole cell patch-clamp recordings were obtained from ventral EGFP+ neurons in spinal cord slices (T1–L2, 200–250 μm). Ih was found in 51% of EGFP+ neurons (n = 149) with almost even distribution in lamina VII (51%), VIII (47%), and X (55%). Ih could be blocked by ZD7288 (10–20 μM) or cesium (1–1.5 mM) but was insensitive to barium (2–2.5 mM). Ih activated at −80.1 ± 9.2 mV with half-maximal activation −95.5 ± 13.3 mV, activation rate 10.0 ± 3.2 mV, time constant 745 ± 501 ms, maximal conductance 1.0 ± 0.7 nS, and reversal potential −34.3 ± 3.6 mV. 5-HT (15–20 μM) and ACh (20–30 μM) produced variable effects on Ih, 5-HT increased Ih in 43% of EGFP+ neurons (n = 37), decreased Ih in 24%, and had no effect on Ih in 33% of the neurons. ACh decreased Ih in 67% of EGFP+ neurons (n = 18) with unchanged Ih in 33% of the neurons. This study characterizes the Ih in locomotor-related interneurons and is the first to demonstrate the variable effects of 5-HT and ACh on Ih in rodent spinal interneurons. The finding of 5-HT and ACh-induced reduction of Ih in EGFP+ neurons suggests a novel mechanism that the motor system could use to limit the participation of certain neurons in locomotion.
regulation of neuronal excitability and performance of functional roles in various systems. In the spinal motor system, however, these features have never been studied in spinal interneurons related to locomotion. In the present study, we investigate the effect of 5-HT on \( I_h \) in EGFP+ neurons and explore the putative mechanisms mediating the effect.

Like 5-HT, ACh is another essential neurotransmitter for generation of locomotion. Both transmitters have been shown to produce locomotor-like activity in isolated spinal cords of neonatal rats and mice (Cowley and Schmidt 1994; Jiang et al. 1999; Smith and Feldman 1987). In contrast to 5-HT, however, our knowledge about ACh modulation of \( I_h \) in rodent spinal neurons is very limited. In this study, we investigate the effect of ACh on \( I_h \) in EGFP+ neurons. Preliminary data of this study have been reported in abstract form (Dai et al. 2005a).

**Methods**

The animal protocol was approved by the University of Manitoba Central Animal Care Services and conformed to the standards of the Canadian Council on Animal Care.

**Preparation of slices and patch-clamp recordings**

Cfos-EGFP transgenic mice, produced as described in our recent study (Dai et al. 2009), were used in this study. Experiments were carried out on neonatal (P6-12) cfos-EGFP mice. A locomotor task (swimming) was induced in the animals prior to preparation of slices. A length of 1–3 cm wide paper tape was prepared for suspending the animal by making the portion of tape that touches the animal non-adhesive with a 4–6 cm long piece of the same tape placed sticky side to sticky side. This tape was then placed around the animal’s thorax, and the distal ends were joined over the animal’s back, leaving ample length of tape to allow the attachment of a clamp for suspension of the animal over a beaker of water. The tape allowed the mouse full range of motion in all limbs as well as normal breathing. The ends of the tape were placed in a clamp attached to a calibrated microdrive. The water bath was held at 26–30°C (usually 28°C) using a hotplate and thermometer. The microdrive was used to lower the mouse into the water bath. This “swim” procedure for the preparation of spinal cord slices was similar to that previously reported (Dai et al. 2009). Briefly, after the locomotion task, the mice were anesthetized with an injection of ketamine (100 mg/kg ip). The spinal cords were then dissected. Slices were cut at thickness of 250–300 \( \mu \)m from T12 to L4 segments and remained in the recording artificial cerebrospinal fluid (ACSF) for \( \approx 1 \) h before the patch-clamp recordings. The slices were then transferred to a recording chamber mounted in the stage of an upright Olympus BX50 microscope fitted with differential interference contrast (DIC) optics and epifluorescence. The chamber was perfused with recording ACSF at rate of 2 ml/min, bubbled with 95% \( \text{O}_2 \)-5% \( \text{CO}_2 \). The EGFP-positive neurons were identified at \( \times 40 \) magnification using epifluorescence with a narrow band GFP cube. The neurons were then visualized using an infrared cube and patched using glass pipette electrodes. Lamina VII, VIII, and X were set as the target area in this study. Only neurons in this area were selected for patch-clamp recordings (Dai et al. 2009). The pipette electrodes were pulled from borosilicate glass (WPI, MT50F-4) using an electrode puller (P-87, Sutter Instrument) and had resistances of 5–8 \( \Omega \) when filled with intracellular solution. A MultiClamp 700A patch-clamp amplifier, Digidata 1322A A/D converter, Minidigit 1A, and pClamp (9.0) software (all from Molecular Devices) were used for data acquisition. Data were low-filtered at 3 kHz and sampled at 10 kHz. Whole cell patch recordings were made in voltage-clamp mode with series resistance compensation by 70–80% and in current-clamp mode with bridge balance and capacitance compensation.

**Measurement of \( I_h \) current and sag voltage**

The \( I_h \) was recorded through a family of 1–3 s step voltages with step of \(-5 \) or \(-10 \) mV, peak voltage of \(-120 \) to \(-160 \) mV, and holding potential of \(-40 \) to \(-60 \) mV (Fig. 1 A1). \( I_h \) is defined as the current difference between the instantaneous current (\( I_{\text{ss}} \)) and steady state inward current (\( I_{\text{iis}} \)), that is, \( I_h = I_{\text{ss}} - I_{\text{iis}} \) (Fig. 1A2). The step voltage at which the first \( I_h \) was elicited was defined as the activation voltage of the \( I_h \) and denoted as \( V_{\text{act}} \). The largest \( I_h \) elicited in the last three steps was fitted with a single exponential function \( f(t) = I_h \exp(-t/\tau) \) to determine the time constant \( \tau \) (Fig. 1A2). In a few of neurons, a double exponential function was used to determine the \( \tau \). Conductance of \( I_h (G_h) \) at each step was calculated with the formula \( G_h = \frac{I_h}{V - V_{\text{act}}} \), where the \( V \) was step voltage. The maximal conductance (\( g_{\text{max}} \)) was defined as the maximal \( G_h \), that is, \( g_{\text{max}} = \max [G_{\text{hi}}, i = 1, \ldots, n] \). The voltage dependency of \( G_h \) was constructed by fitting the Boltzmann equation \( f(V) = \frac{1}{\cosh \left( \frac{V - V_{\text{act}}}{V_{\text{act}}} \right)} \) to the normalized I-V curves generated from the tail currents (or \( I_h \) currents in some neurons wherever the tail currents is not available), where the \( V_{\text{act}} \) is half-maximal activation of \( I_h \) and \( V \) is the voltage that determines the rate of activation. To get the best-fitting of the Boltzmann equation, extrapolating points were used in some neurons. The depolarizing sag voltage was recorded through a family of 1-s step currents with step of \(-50 \) pA (Fig. 1B1). The sag was defined as the voltage difference between the instantaneous voltage (\( V_{\text{ss}} \)) and steady state voltage (\( V_{\text{ss}} \)), that is, \( \text{sag} = V_{\text{ss}} - V_{\text{ss}} \) (Fig. 1B2).

During the experiment, the recording protocols were repeated three to five times in each condition (control, drugs, and washout etc.) with 30–40 s between two successive recordings. Normally recordings were made 2–8 min following drug application and repeated for 10–15 min before switching to new conditions. The data were averaged from 2–5 trials for measurement of \( I_h \) parameters. Recordings were made for one neuron per slice.

**Solutions and chemicals**

**EXTRACELLULAR SOLUTIONS.** The dissecting ACSF contained (in mM) 25 NaCl, 188 sucrose, 1.9 KCl, 1.2 NaHPO\(_4\), 10 MgSO\(_4\), 26 NaHCO\(_3\), 1.0 CaCl\(_2\), 1.5 kynurenic acid, and 25 glucose. The recording ACSF contained (in mM) 130 NaCl, 4.5 KCl, 1.25 NaHPO\(_4\), 1.25 MgCl\(_2\), 26 NaHCO\(_3\), 2.5 CaCl\(_2\), and 10 glucose. The pH of these solutions was \( \approx 7.3 \) when bubbled with 95% \( \text{O}_2 \)-5% \( \text{CO}_2 \).

**INTRACELLULAR SOLUTIONS.** The intracellular solution contained (in mM) 120 potassium gluconate, 5 NaCl, 10 HEPES, 5 EGTA, 2 MgCl\(_2\), 1 CaCl\(_2\), 5 Mg-ATP, and 0.5 GTP. pH adjusted to 7.3 with...
KOH. Osmolarity was adjusted to 310 mosM by adding sucrose to the solution. All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

BLOCKERS. Recordings were made with 2-amino-5-phosphonovaleric acid (APV, 20 μM), 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 10 μM), bicuculline (10 μM), and strychnine (10 μM) in the recording solutions. In most experiments, TTX (1 μM) was used to block the transient sodium currents and TEA (10 mM) and 4-amino-pyridine (4-AP, 4 mM) were used to block potassium currents. Ca\(^{2+}\) was replaced by Ba\(^{2+}\) with the same concentration in some experiments. All the recordings were made at room temperature (20 – 22°C).

LIQUID JUNCTION POTENTIAL. The liquid junction potential was calculated as 10.4 mV with pH value of recording solution adjusted to 7.3 by KOH, osmolarity adjusted to 310 mosM by sucrose, and presence of 10 mM TEA in the solution. This value was not corrected in this study to make our results comparable to those from previous studies (Butt et al. 2002; Kjaerulff and Kiehn 2001).

RESULTS

We demonstrated previously that depolarizing sag induced by \(I_h\) was observed in about half of the EGFP+ neurons recorded with current-clamp protocol (Dai et al. 2009). About half of the neurons that displayed sag possessed the property of postinhibitory rebound. Due to limitations of the recording protocol, the details of the \(I_h\) property were not explored. In the present study, however, we characterized the \(I_h\) in EGFP+ neurons with a voltage-clamp protocol. The kinetics and pharmacological properties of \(I_h\) were studied in detail, and the modulatory properties of \(I_h\) by 5-HT and ACh were also investigated.

\(I_h\) in EGFP+ neurons

Consistent with our recent report (Dai et al. 2009), \(I_h\) is widely distributed in EGFP+ neurons. Figure 2 shows four examples of \(I_h\) recorded in current- and voltage-clamp protocols with or without postinhibitory rebound. In current-clamp recordings, \(I_h\) was shown as a depolarizing sag induced by hyperpolarizing step currents (Fig. 2A1). In some neurons, the sag could generate spike(s) following the termination of the step currents (Fig. 2A2). \(I_h\) could be also shown as a slow inward current induced by hyperpolarizing step voltages in voltage-clamp recordings (Fig. 2B1). A transient spike could be elicited by \(I_h\) in some neurons right after the termination of the step voltages (Fig. 2B2).

A total of 94 EGFP+ neurons were recorded with voltage-clamp protocol (Fig. 2C). \(I_h\) was observed in 51% of the neurons (48/94) with almost even expression in lamina VII (49%, 18/37), VIII (50%, 10/20), and X (54%, 20/37). Postinhibitory rebound was observed in 36% of the neurons that displayed \(I_h\) (10/28). In another total of 55 EGFP+ neurons recorded with current clamp (Fig. 2D), sag was observed in 53% of the neurons (29/55) with 55% in lamina VII (11/20), 43% in VIII (6/14), and 57% in X (12/21). Postinhibitory rebound was observed in 38% of neurons that displayed sag (11/29). In summary, \(I_h\) and sag were seen in 52% of EGFP+ neurons (77/149) with 51% in lamina VII (29/57), 47% in VIII (16/34), and 55% in X (32/58).

Blockade of \(I_h\) with ZD7288 and cesium

One of the common properties of \(I_h\) is blockade of this current with the antagonists ZD7288 and cesium (Pape 1996; Robinson and Siegelbaum 2003 for review). This property was demonstrated in EGFP+ neurons. In general, \(I_h\) could be completely removed by 10–20 μM ZD7288 (n = 4, Fig. 3A). In some neurons, however, a lower concentration of ZD7288 (~5 μM) was enough to block \(I_h\) (n = 2). Similarly, \(I_h\) was...
and then returned to a 1-s succession of step voltages from amplitude of deactivated

was applied to the blockade of potassium and 157 mM sodium (see METHODS for details). The reversal potential of $I_h$ was estimated from the tail currents using the same method employed in previous studies (Kiehn and Harris-Warrick 1992; Kjaerulff and Kiehn 2001; Takahashi 1990). In the present study, we recorded $I_h$ in normal ACSF with concentrations of 4.5 mM potassium and 157 mM sodium (see METHODS for details). The reversal potential was estimated from the tail currents using the same method employed in previous studies (Kiehn and Harris-Warrick 1992; Kjaerulff and Kiehn 2001; Takahashi 1990).

Reversal potential ($E_{rh}$) of $I_h$

$I_h$ is mediated by mixed sodium and potassium currents. Therefore the reversal potential of $I_h$ ($E_{rh}$) is dependent on the extracellular concentrations of potassium and sodium (Kjaerulff and Kiehn 2001; Takahashi 1990). In the present study, we recorded $I_h$ in normal ACSF with concentrations of 4.5 mM potassium and 157 mM sodium (see METHODS for details). The reversal potential was estimated from the tail currents using the same method employed in previous studies (Kiehn and Harris-Warrick 1992; Kjaerulff and Kiehn 2001; Takahashi 1990). TTX (1 μM) was applied to the recording solution to block the spikes elicited from postinhibitory rebound. The membrane potential was hyperpolarized to −120 mV for 3 s from the holding potential of −60 mV (or −40 mV in some neurons) and then returned to a 1-s succession of step voltages from −80 to −20 mV with a step of 5 or 10 mV (see Fig. 3C, inset). The amplitude of deactivated $I_h$ (tail current) was plotted against the step voltage to construct the $I-V$ curve. A linear regression was applied to the $I-V$ curve, and the $E_{rh}$ was estimated as the potential at which the straight line intersected with the voltage axis where the tail current was zero. Results from five neurons are shown in Fig. 3C. For clarity, only the linear regression for averaged $I-V$ curve is shown in the figure. The reversal potentials of $I_h$ for the five neurons varied from −29.5 to −35.8 mV, and the mean value of the $E_{rh}$ was −34.3 ± 3.6 mV. This value was comparable to that reported in spinal motoneurons in previous studies (Kjaerulff and Kiehn 2001; Takahashi 1990).

General properties of $I_h$ in EGFP+ neurons

We measured a set of parameters for description of $I_h$ properties. These parameters included the voltage dependency, time constant, and maximal conductance. The kinetics of the $I_h$ was characterized by fitting the Boltzmann equation to the normalized $I-V$ curves (see METHODS for details). Results from 38 EGFP+ neurons showed that the $I_h$ was activated at −80.1 ± 9.2 mV with half-maximal activation ($V_{mid}$) of −95.5 ± 13.3 mV and activation rate ($V_C$) of 10.0 ± 3.2 mV (Fig. 3D1). $I_h$ had a time constant ($\tau$) of 745 ± 501 ms and maximal conductance ($g_{max}$) of 1.0 ± 0.7 nS. In general, these values of the parameters are similar to those reported in spinal descending commissural interneurons (Butt et al. 2002) but different from (generally lower than) those in spinal motoneurons (Kjaerulff and Kiehn 2001; Kiehn et al. 2000). A further analysis uncovered a broad range of these parameters. The $V_{mid}$ was distributed from −120 to −70 mV with a range of −110
to −90 mV in most of the neurons (58%, 22/38, Fig. 3D2). A few neurons (<15%) had a \( V_{\text{mid}} \) higher than −80 mV or lower than −110 mV. The distributions of \( g_{\text{max}} \) (Fig. 3D3) and \( \tau \) (Fig. 3D4) were positively skewed. Most of neurons had a \( g_{\text{max}} \) < 1.0 nS (66%, 25/38) and a \( \tau \) < 1.0 s (76%, 29/38). The wide range and variable values of \( I_h \) parameters reflected the difference of membrane properties in the heterogeneous population of the neurons recorded in this study. Table 1 summarizes the properties of \( I_h \) recorded from 38 EGFP+ neurons with laminar distribution. Except for some small variation, the properties of \( I_h \) appeared to be evenly distributed in lamina VII, VIII, and X. No significant difference was found between the \( I_h \) properties and laminar distribution of these neurons.

Sags recorded with a family of hyperpolarizing step currents (see METHODS) from 29 EGFP+ neurons are summarized in Table 2. The resting membrane potential (\( E_m \)) and input resistance (\( R_{\text{in}} \)) are also shown in the table because \( I_h \) potentially contributes to regulation of these two properties. Sags increased with hyperpolarization of the step currents in all neurons crossing lamina VII, VIII, and X. In total of 29 neurons, sags were measured as 4.5 ± 5, 6.5 ± 4, 9.4 ± 5, and 12.4 ± 7 mV with respect to steps of −50, −100, −150, and −200 pA, respectively. Similar to the case of \( I_h \) there was no significant difference between the sags and laminar distribution. Neither did the membrane potential show a significant difference with laminar distribution. However, the input resistance was found significantly different (\( P < 0.05 \)) in lamina VII, VIII, and X neurons, similar to the observation in our recent report (Dai et al. 2009).

**Modulation of \( I_h \) by 5-HT**

One of the important properties of \( I_h \) is its ability to be modulated by neurotransmitters such as 5-HT. In spinal neu-

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**TABLE 1.** \( I_h \) with laminar distribution (n = 38)

<table>
<thead>
<tr>
<th>Laminae</th>
<th>( V_{\text{rest}} ) mV</th>
<th>( V_{\text{mid}} ) mV</th>
<th>( V_{\text{c}} ) mV</th>
<th>( \tau ) ms</th>
<th>( g_{\text{max}} ) nS</th>
</tr>
</thead>
<tbody>
<tr>
<td>VII</td>
<td>−79.2 ± 9</td>
<td>−95.1 ± 15</td>
<td>9.6 ± 3</td>
<td>745.9 ± 449</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>VIII</td>
<td>−78.6 ± 7</td>
<td>−93.9 ± 10</td>
<td>9.7 ± 3</td>
<td>691.3 ± 394</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>X</td>
<td>−81.4 ± 9</td>
<td>−96.4 ± 12</td>
<td>10.5 ± 4</td>
<td>765.9 ± 590</td>
<td>0.9 ± 0.9</td>
</tr>
<tr>
<td>Mean</td>
<td>−80.1 ± 9</td>
<td>−95.5 ± 13</td>
<td>10.0 ± 3</td>
<td>745.3 ± 501</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>( p )</td>
<td>0.7</td>
<td>0.9</td>
<td>0.7</td>
<td>0.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>

\( * \), \( P \) values of single factor ANOVA for laminar (VII, VIII, and X) distribution of the selected properties.
Ih has been shown to be enhanced by 5-HT in many studies. However, the effects of 5-HT on Ih could be variable with types of cells and systems. In the present study, we demonstrate that activation of serotonergic receptors in EGFP+ neurons results in varied effects on Ih.

Ih COULD BE ENHANCED BY 5-HT. 5-HT enhancement of Ih has usually been described as a depolarization of voltage activation and/or an increase in conductance. In the present study, we demonstrate that activation of serotonergic receptors in EGFP+ neurons results in varied effects on Ih.

**TABLE 2. Sags with laminar distribution (n = 29)**

<table>
<thead>
<tr>
<th>Laminae</th>
<th>$E_r$, mV</th>
<th>$R_m$, MΩ</th>
<th>Sags measured at each step current (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VII</td>
<td>$n = 11$</td>
<td>$-64.9 \pm 7$</td>
<td>273.9 ± 83</td>
</tr>
<tr>
<td>VIII</td>
<td>$n = 6$</td>
<td>$-69.6 \pm 8$</td>
<td>327.6 ± 128</td>
</tr>
<tr>
<td>X</td>
<td>$n = 12$</td>
<td>$-68.9 \pm 7$</td>
<td>446.0 ± 243</td>
</tr>
<tr>
<td>Mean</td>
<td>$n = 29$</td>
<td>$-67.6 \pm 7$</td>
<td>360.9 ± 191</td>
</tr>
</tbody>
</table>

$E_r$, resting membrane potential; $R_m$, input resistance. *, P values of single factor ANOVA for laminar (VII, VIII, and X) distribution of the selected properties.

The enhancement of Ih was also demonstrated in Ih-mediated sag recorded with current-clamp protocol. A typical example is
shown in Fig. 5, where the sags were recorded by a family of hyperpolarizing step currents with step of −50 pA (A1). Bath application of 30 μM 5-HT increased the sags (Fig. 5A2) in a voltage dependent manner (Fig. 5A3) similar to that observed in \( I_h \) (Fig. 4A2). In a total of 15 EGFP+ neurons recorded with current-clamp protocol, 5-HT-enhancement of sag (i.e., increase in the maximum sag recorded in the last current step >2 mV) was observed in 40% of the neurons (6/15). This number is consistent with that observed in \( I_h \). The voltage-dependent enhancement of sag by 5-HT from the six neurons is shown in Fig. 5B.

\( I_h \) COULD BE REDUCED BY 5-HT. One of the remarkable findings in this study was 5-HT induced reduction of \( I_h \). A similar observation was reported in previous studies in spinal neurons (Kjaerulff and Kiehn 2001; Takahashi and Berger 1990). But this observation was described as a time-dependent rundown of 5-HT effect on \( I_h \), which was related to a current modulated by diffusible second messengers. To make sure that our data were not contaminated by this rundown effect, we chose recordings restricted by the following two constrains: 1) the reduction of \( I_h \) was observed immediately (3–5 min) after bath administration of 5-HT (15–20 μM); 2) the neurons were healthy for the recordings in the entire period of time with a full or partial recovery obtained after washout (15–30 min). Two examples of 5-HT-induced reduction of \( I_h \) are shown in Fig. 6. Figure 6A shows the \( I_h \) evoked by a succession of step voltages with holding potential of −50 mV. The maximal \( I_h \) of ~50 pA was evoked at the last step of −130 mV (Fig. 6A1). Bath application of 15 μM 5-HT reduced \( I_h \) by 60% to ~20 pA. The \( I_h \) partially recovered (~40%) after a 15 min washout. The whole range of 5-HT-induced reduction of \( I_h \) is shown in the \( I-V \) curves in Fig. 6A2. 5-HT reduced \( I_h \) by >50% in the whole voltage range of the recordings, and ~35% recovery of \( I_h \) was obtained after washout. 5-HT also induced hyperpolarization of voltage dependency in this neuron (Fig. 6A3). The half-maximal activation (\( V_{mid} \)) was hyperpolarized by 4.8 mV from control (~97.6 mV) and activation rate (\( V_c \)) was reduced by 0.7 from 9.5 mV. The time constant of \( I_h \) was increased by

### Table 3. 5-HT-induced changes in \( I_h \) properties

<table>
<thead>
<tr>
<th>Changes in ( g_{max} )</th>
<th>( V_{act} ), mV</th>
<th>( V_{mid} ), mV</th>
<th>( V_c ), mV</th>
<th>( \tau ), ms</th>
<th>( g_{max} ), nS</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta g_{max} &gt; 0 ) (10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>−78.8 ± 8</td>
<td>−91.4 ± 13</td>
<td>10.3 ± 4</td>
<td>556.4 ± 181</td>
<td>1.1 ± 0.9</td>
</tr>
<tr>
<td>Changes by 5-HT</td>
<td>3.1 ± 4†</td>
<td>1.0 ± 3</td>
<td>0.1 ± 2</td>
<td>−75.5 ± 62†</td>
<td>0.4 ± 0.2*</td>
</tr>
<tr>
<td>( \Delta g_{max} &lt; 0 ) (5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>−83.9 ± 9</td>
<td>−100.1 ± 16</td>
<td>9.7 ± 4</td>
<td>672.3 ± 297</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Changes by 5-HT</td>
<td>−2.8 ± 7†</td>
<td>−1.1 ± 5</td>
<td>−0.8 ± 2</td>
<td>114 ± 309</td>
<td>−0.3 ± 0.1**</td>
</tr>
<tr>
<td>( \Delta g_{max} = 0 ) (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>−80.1 ± 7</td>
<td>−88.8 ± 17</td>
<td>9.4 ± 3</td>
<td>434.5 ± 144</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Changes by 5-HT</td>
<td>0.0 ± 0.5</td>
<td>0.3 ± 1</td>
<td>−0.8 ± 2</td>
<td>11.8 ± 81</td>
<td>0.01 ± 0.1</td>
</tr>
<tr>
<td>Mean for all (22)</td>
<td>−81.1 ± 8</td>
<td>−94.4 ± 15</td>
<td>9.9 ± 4</td>
<td>576.1 ± 239</td>
<td>0.9 ± 0.7</td>
</tr>
<tr>
<td>Changes by 5-HT</td>
<td>0.0 ± 5</td>
<td>−0.6 ± 4</td>
<td>−0.2 ± 2</td>
<td>21.8 ± 215</td>
<td>0.01 ± 0.3</td>
</tr>
</tbody>
</table>

\( n \) values in parentheses. †, \( P < 0.05; * \), \( P < 0.005; ** \), \( P < 0.0001 \). \( \Delta g_{max} \), changes induced by 5-HT in \( I_h \) maximal conductance.

**Fig. 5.** Enhancement of \( I_v \)-mediated sag by 5-HT. A: sags were recorded by a family of hyperpolarizing currents with step of −50 pA in an EGFP+ neuron (A1). Bath application of 30 μM 5-HT increased the sags (A2) with ~4 mV depolarization of the membrane potentials. The sag was increased with hyperpolarization of step currents and the current-sag curves were well fitted by straight lines in both conditions in this neuron (A3). B: results of 5-HT-enhancement of sag from 6 EGFP+ neuron. The enhancement was defined as the increase >2 mV in the maximum sag recorded in the last current step.
FIG. 6. Reduction of $I_h$ by 5-HT. A1: $I_h$ was evoked by a succession of step voltages with holding potential of $-50 \text{ mV}$, step of $10 \text{ mV}$, and duration of $3 \text{ s}$ in 3 sequent conditions: control, 5-HT (15 $\mu$M), and washout (15 min). Traces from the last step of the 3 conditions were overlapped to show the difference in $I_h$. A2: $I-V$ curves were plotted for $I_h$ measured from the three conditions. A3: the normalized $I-V$ curves were fitted by Boltzmann equation, and the parameters $V_{\text{mid}}$ and $V_c$ were obtained from the equation: control: $V_{\text{mid}} = -97.6 \text{ mV}, V_c = 9.5 \text{ mV}$; 5-HT: $V_{\text{mid}} = -102.4 \text{ mV}, V_c = 8.8 \text{ mV}$; and washout: $V_{\text{mid}} = -102.1 \text{ mV}, V_c = 9.3 \text{ mV}$. The time constant for $I_h$ activation was increased by 5-HT from 837.9 to 1,177.4 ms and partially recovered to 995.3 ms after washout. 5-HT reduced $I_h$ with reduction of maximal conductance more observable than the hyperpolarization of voltage dependency. B: similar to the preceding example $I_h$ was evoked by a family of step voltages with holding potential of $-40 \text{ mV}$, step of $-5 \text{ mV}$, and duration of $3 \text{ s}$ in another EGFP+ neuron in 3 sequent conditions: control (B1), bath application of 25 $\mu$M 5-HT (B2), and 20 min washout (B3). The $I-V$ curves were plotted for $I_h$ measured in the 3 conditions (B4), which show that the recovery of $I_h$ from washout was voltage dependent. It partially recovered within the step voltages from $-75$ to $-90 \text{ mV}$, fully recovered from $-95$ to $-100 \text{ mV}$, and increased in the last three steps from $-105$ to $-115 \text{ mV}$. Inset: overlapped traces of $I_h$ evoked in the last step from the 3 conditions. Dashed curves were single exponential functions fitted to the $I_h$ traces in control (middle), 5-HT (top), and washout (bottom). 5-HT increased time constant by 44.4 ms from 401.9 to 446.3 ms, and washout did not get it recovered but further increased it to 554.8 ms. C: results of reduction of $I_h$ by 5-HT from 5 EGFP+ neurons. The 5-HT-induced reduction of $I_h$ was observed in 23% of the neurons (5/22). It was shown as a significant reduction of maximal conductance by $-43\%$ and a hyperpolarization of $I_h$ activation voltage by $-3\%$. 5-HT also induced a decrease in both half-maximal activation ($-1\%$) and rate ($-8\%$) and an increase in time constant ($17\%$). However, these changes in $V_{\text{mid}}, V_c,$ and $\tau$ were not statistically significant. See text for details.
displayed a reduction of \( \tau (~7\%) \) with 5-HT. Therefore the increase of time constant was not shown to be statistically significant. See Fig. 6C and Table 3 (\( \Delta g_{max} < 0 \)) for details.

The reduction of \( I_h \) by 5-HT was also observed in \( I_h \)-mediated sag recorded with current-clamp protocol. An example is shown in Fig. 7. Three sags were evoked by family of step currents in an EGFP+ neuron (Fig. 7A1). Bath application of 20 \( \mu M \) 5-HT almost completely removed the sags from this neuron (Fig. 7A2). However, sag was recovered by \( >90\% \) in the third step after 15 min washout (Fig. 7A3). 5-HT did not induce substantial changes in membrane potential and input resistance in this neuron but completely blocked the sags in the first two steps and reduced the sag by \( >70\% \) in the third step (Fig. 7A4). Of 15 EGFP+ neurons recorded with current clamp, the 5-HT-induced reduction of sag was observed in 4 of them (\( ~50\% \) reduction on average, Fig. 7B), accounting for 27% of the neurons. This number is comparable to that observed in \( I_h \) (Fig. 6C).

\( I_h \) DID NOT RESPOND TO 5-HT. While modulation of \( I_h \) by 5-HT (either enhancement or reduction of \( I_h \)) was observed in 68% of the neurons (15/22), \( I_h \) with no response to 5-HT (15–20 \( \mu M \)) was still shown in 32% of the neurons (7/22). We defined this null effect of 5-HT on \( I_h \) as a change induced by 5-HT in \( g_{max} <5\% \) and in voltage dependency (\( V_{act} \) and \( V_{mid} \)) \( <1 \) mV. Recordings from seven neurons are shown in Table 3 (\( \Delta g_{max} = 0 \)). Except for some small variations, \( I_h \) did not respond to 5-HT in these neurons. The null effect of 5-HT on \( I_h \) was also observed in sag. This was defined as the 5-HT induced changes in sag \( <2 \) mV. In a total of 15 EGFP+ neurons recorded with current clamp, 33% of the neurons (5/15) did not respond to 5-HT. This number is similar to that for \( I_h \) in voltage-clamp recordings. A similar observation was also reported in ascending commissural interneurons in a recent study (Zhong et al. 2006a).

Mechanisms underlying the modulation of \( I_h \) by 5-HT

The present study demonstrated the varied effect of 5-HT on \( I_h \). It is unclear what mechanism responsible for this phenomenon. We first thought this variable modulation of \( I_h \) might be related to the neuronal lamina distributions. We then classified the neurons into their laminar locations and looked at the effect of 5-HT on \( I_h \) in these neurons. The results are shown in Table 4. These results indicate that the variable effects of 5-HT on \( I_h \) or sag are widely observed in the neurons crossing lamina VII (\( n = 14 \)), VIII (\( n = 8 \)), and X (\( n = 15 \)). Statistical analysis with \( x^2 \) test shows that there is no significant correlation between the 5-HT effects and neuronal distributions (\( P = 0.6 \)). Positive effects of 5-HT on \( I_h \) (i.e., \( \Delta g_{max} > 0 \) or Sag \( >2 \) mV) was observed in 50% of the neurons in lamina X, 31% in VII, and 19% in VIII. But this preference for lamina X was not statistically significant (\( P = 0.3 \)) with respect to the positive effects of 5-HT on laminae VII and VIII neurons.

Because the neurons we recorded were a heterogeneous population, they could play different functional roles during locomotion. This difference might be reflected more from their modulatory properties than their lamina distributions. 5-HT enhancement of \( I_h \) has been previously shown to be mediated by 5-HT7 receptors in rat dorsal root ganglion neurons (Cardenas et al. 1999) and anterodorsal thalamus (Chapin and Andrade 2001a,b). Based on these studies, therefore we did some additional experiments to test the 5-HT7 receptors in modulation of \( I_h \). In contrary to our expectation, however, our results did not suggest a role of 5-HT7 receptors in mediating the enhancement of \( I_h \) in EGFP+ neurons. An example is

![Figure 7](http://jn.physiology.org/)

**TABLE 4.** Lamina distribution with 5-HT effect on \( I_h \) and sag

<table>
<thead>
<tr>
<th>5-HT effect on ( I_h ) (( n = 22 )) and Sag (( n = 15 ))</th>
<th>Laminae</th>
<th>Total Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta g_{max} &gt; 0 )</td>
<td>VII</td>
<td>3</td>
</tr>
<tr>
<td>( \Delta g_{max} &lt; 0 )</td>
<td>VIII</td>
<td>2</td>
</tr>
<tr>
<td>( \Delta g_{max} = 0 )</td>
<td>X</td>
<td>1</td>
</tr>
<tr>
<td>( \Delta Sag &gt; 2 ) mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta Sag &lt; 2 ) mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta Sag = 0 )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( *\Delta Sag = 0 \) was defined as \( \pm 2 \) mV.
shown in Fig. 8. \(I_h\) was measured as \(-50.6\, \text{pA}\) at step voltage of \(-120\, \text{mV}\) in control (Fig. 8A, black trace). Bath application of 20 \(\mu M\) 5-HT enhanced \(I_h\) by \(-20\, \text{pA}\) at the same step voltage (Fig. 8A, red trace). This enhancement of \(I_h\) was not altered by a following application of 20 \(\mu M\) SB269970, an antagonist of 5-HT\(_7\) receptors (Fig. 8A, blue trace). However, \(I_h\) could be completely blocked by a 15 \(\mu M\) ZD7288 (Fig. 8A, green trace). Details of the whole range recordings are described in the \(I-V\) curves in Fig. 8B. The null effect of SB269970 (20–25 \(\mu M\)) on the 5-HT-induced enhancement of \(I_h\) was observed in three neurons tested for this study. These results suggested that the 5-HT-induced enhancement of \(I_h\) might not be necessarily mediated by 5-HT\(_7\) receptors alone in EGFP+ neurons.

**Modulation of \(I_h\) by ACh**

One of important parts of this study is investigation of ACh modulation of \(I_h\) in EGFP+ neurons. ACh-induced reduction of \(I_h\) is observed in our recent study (Dai et al. 2009). However, details of this reduction remain unknown. Furthermore, we find little information available about ACh modulation of \(I_h\) in rodent spinal neurons. In this study, therefore we tested the effect of ACh on \(I_h\) in EGFP+ neurons. Eighteen EGFP+ neurons were recorded with ACh (20–40 \(\mu M\)). Our results indicate that activation of cholinergic receptors reduced \(I_h\)-mediated sag. An example is shown in Fig. 9A. Two sags were induced by the current steps (Fig. 9A1, inset) with 9.1 mV for the first sag and 17.8 mV for the second one (Fig. 9A1, top) in

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**FIG. 8.** A test of 5-HT\(_7\) receptors in mediating enhancement of \(I_h\): \(I_h\) was evoked by a succession of step voltages with holding potential of \(-50\, \text{mV}\), step of \(-10\, \text{mV}\), and duration of 3 s in 4 sequent conditions: \(I_h\) was evoked in control; \(I_h\) was enhanced by bath application 15 \(\mu M\) 5-HT; bath application of SB269970, antagonist of 5-HT\(_7\) receptors, did not substantially alter the 5-HT-induced enhancement of \(I_h\); and a 15 \(\mu M\) ZD7288 completely removed \(I_h\). Traces from the last step of the 4 conditions were overlapped to show the changes in \(I_h\) (black for control, red for 5-HT, blue for SB269970, and green for ZD7288). B: \(I-V\) curves were plotted for \(I_h\) measured from the 4 conditions. See text for details.
control. Both sags were reduced by >72% (−6.6 mV for the 1st and −12.9 mV for the 2nd) accompanying reduction of input resistance after bath application of 30 μM ACh (Fig. 9A1, middle). The reduction of the second sag was partially recovered by ∼70% from control after 30 min washout (Fig. 9A1, bottom). The current-sag curves explore the details of the ACh-induced reduction of sag in the whole range of step currents (Fig. 9A2). Note that the second sag (4.9 mV) measured in ACh was ∼4.2 mV less than the first sag (9.1 mV) measured in control (Fig. 9A2) while the membrane potential for the second sag in ACh was more hyperpolarized (more than −5 mV in average) than that for the first sag in control. This result suggests that the ACh-induced reduction of sag is not related to the reduction of input resistance. Eight of 12 EGFP+ neurons displayed the reduction of sag by ACh. In some neurons, however, ACh could completely block sag. An example is shown in Fig. 9B, where sags (Fig. 9B1, top) were induced by a family of four step currents (Fig. 9B1, inset) in another EGFP+ neuron. A 25 μM ACh completely removed sags from this neuron (Fig. 9B1, bottom) in whole range of the current steps (B2). The ACh blockage of sag was observed in 4 of 12 EGFP+ neurons. Of 18 EGFP+ neurons, the ACh-induced reduction or blockage of sag was observed in 67% of the neurons (12/18). The remaining 33% of the neurons (6/18) did not respond to ACh in sags (i.e., changes in sag <2 mV). No enhancement of sag by ACh was observed in this study. The averaged results from 12 EGFP+ neurons are shown in Fig. 9C. These results indicate that ACh significantly (P < 0.005) reduces the sag by >60% on average in EGFP+ neurons.

**Discussion**

In this study, we investigated the hyperpolarization-activated inward current (I_h) in spinal interneurons that were activated by locomotor activity. We characterized the I_h with its electrophysiological and pharmacological properties. Furthermore, we studied the modulatory properties of I_h by 5-HT and ACh. Our study showed that activation of 5-HT receptors in EGFP+ neurons produced variable effects on I_h whereas activation of cholinergic pathway mainly reduced I_h-mediated sag in EGFP+ neurons.

**Comparison of I_h properties in EGFP+ neurons with other spinal neurons**

I_h in EGFP+ neurons could be completely blocked by bath application of 10–20 μM ZD7288 or 1–1.5 mM cesium. But I_h was insensitive to barium (2–2.5 mM). The biophysical parameters of I_h measured in EGFP+ neurons are generally in agreement with those reported previously in spinal motoneurons (Kjaerulff and Kiehn 2001; Takahashi 1990) and descending commissural interneurons (dCINs) (Butt et al. 2002). For comparison, we summarize the I_h properties measured in different spinal neurons and preparations in Table 5. From this table we can see that the kinetics and reversal potentials of I_h are very similar in these neurons, suggesting that the I_h is not distinguishable in its primary properties from neuron to neuron in spinal motor system. Instead any functional differences may be related to the functional roles of the neurons, and perhaps more importantly, those roles attributable to I_h can be modulated by neurotransmitters such as 5-HT and ACh.

Despite the preceding similarities, however, the I_h were also shown to be different in g_max and V_act between EGFP+ neurons and other spinal neurons. First, the mean value of g_max in EGFP+ neurons was >10 times less than that in spinal motoneurons (Kjaerulff and Kiehn 2001). Second, a 10 mV difference in V_act was observed between EGFP+ neurons and dCINs (i.e., the activation of I_h in EGFP+ neurons was 10 mV lower than that in dCINs). And third, the V_mid was found to be 10 mV lower in dCINs of slow τ than dCINs of fast τ (Butt et al. 2002), but this difference was not observed in EGFP+ neurons. We do not know what causes these differences in I_h in rodent spinal neurons, but they could result from many factors including the difference in preparations (slices vs. intact cords), neuron types (interneurons vs. motoneurons), and methods for estimation of g_max and V_act. With these unique properties of I_h in EGFP+ neurons, we would expect different functional roles of I_h in the generation of locomotion. However, a further study is required to investigate this issue in the future.

**Variable effects of 5-HT on I_h**

Modulation of I_h by 5-HT has been reported in many types of neurons (Pape 1996; Robinson and Siegelbaum 2003). This modulation is generally shown as an alteration of voltage dependency and/or maximal conductance. Enhancement of I_h by 5-HT is one of the most important properties of I_h. In some neurons, however, inhibition of I_h by 5-HT was also reported (Pape 1996; Robinson and Siegelbaum 2003). In this study, we demonstrated that 5-HT induced variable effects on I_h in EGFP+ neurons. Furthermore, we showed that the variable effect of 5-HT on I_h was not related to the laminar distribution of the neurons.

**TABLE 5. Properties of I_h in rodent spinal neurons**

<table>
<thead>
<tr>
<th>Cell Types &amp; Preparations</th>
<th>V_act, mV</th>
<th>V_max, mV</th>
<th>V_r, mV</th>
<th>τ, ms</th>
<th>g_max, nS</th>
<th>E_rev, mV</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP+ INs (slice)</td>
<td>−80</td>
<td>−96</td>
<td>10.0</td>
<td>745</td>
<td>1.0</td>
<td>−34</td>
<td>Present study</td>
</tr>
<tr>
<td>MNs (slice)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Takahashi (1990)</td>
</tr>
<tr>
<td>[K]_o = 3 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[K]_o = 12 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MNs (cord)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dCINs (cord)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast τ</td>
<td>−70*</td>
<td>−95</td>
<td>11.9</td>
<td>568</td>
<td>0.79</td>
<td>−33</td>
<td>Kjaerulff &amp; Kiehn (2001)</td>
</tr>
<tr>
<td>Slow τ</td>
<td>−70*</td>
<td>−105</td>
<td>11.9</td>
<td>1377</td>
<td>0.79</td>
<td>−33</td>
<td>Butt et. al. (2002)</td>
</tr>
</tbody>
</table>

EGFP, enhanced green fluorescent protein; IN, interneuron; MN, motoneuron; dCIN, descending commissural IN. *Data were estimated from Fig. 8, Takahashi (1990). †Values were equivalent to V_act in this study. They were the thresholds for detection of depolarizing sag in current clamp or slow activating inward current (I_h) in voltage clamp in Butt et al. (2002).
ENHANCEMENT OF \( I_h \) BY 5-HT. 5-HT enhancement of \( I_h \) in EGFP+ neurons was mainly mediated through an increase in the maximal conductance \( (g_{\text{max}}) \) while changes in kinetics \( (V_{\text{mid}} \text{ and } V_j) \) were not substantial (Fig. 4B and Table 3). This modulation was similar to the previous reports from slice recordings of rat spinal motoneurons (Takahashi and Berger 1990) and guinea pig trigeminal motoneurons (Hsiao et al. 1997) but different from the observations in intact cord recordings of rat spinal motoneurons (Kjaerulff and Kiehn 2001) and slice recordings of rat facial motoneurons (Larkman and Kelly 1992), where \( V_{\text{mid}} \) was depolarized with \( g_{\text{max}} \) unchanged. Accompanying a 36% increase in \( g_{\text{max}} \) (Fig. 4B) 5-HT also reduced the time constant for activation of \( I_h \) in EGFP+ neurons. This result agrees with a report in rat spinal motoneurons (Kjaerulff and Kiehn 2001). Although the kinetics of \( I_h \) did not show a significant change by 5-HT in the present study, the activation voltage \( (V_{\text{act}}) \) did go through a 3 mV depolarization, which might result from a mixed effect of a small increase in \( V_a \) and depolarization of \( V_{\text{mid}} \). A 3 mV depolarization of \( V_{\text{act}} \) might not lead to a functional difference in EGFP+ neurons during locomotion. However, this small change in \( V_{\text{act}} \) plus an increase in \( g_{\text{max}} \) could increase the leak inward current from \( I_h \) in the subthreshold range and thus contribute to a resetting of resting membrane potential and input conductance.

5-HT-induced enhancement of \( I_h \) was shown to be mediated by 5-HT\(_7\) receptors in rat dorsal root ganglion neurons (Cardenas et al. 1999) and anterodorsal thalamus (Chapin and Andrade 2001a,b). In spinal motoneurons, however, this enhancement appeared to be mediated by 5-HT\(_{1A}\) and 5-HT\(_2\) receptors (Takahashi and Berger 1990). These studies suggested that the enhancement of \( I_h \) by 5-HT could be mediated by different mechanisms (subfamilies of 5-HT receptors) in different systems. The mechanism underlying 5-HT-induced enhancement of \( I_h \) in EGFP+ neurons remains unknown. Our results, however, did not support the role of 5-HT\(_7\) receptors in this process.

REDUCTION OF \( I_h \) BY 5-HT. A robust finding in this study was 5-HT-induced reduction of \( I_h \) in EGFP+ neurons. This reduction was observed in both voltage clamp for \( I_h \) and current clamp for sag. This modulation of \( I_h \) was shown as a reduction of maximal conductance and hyperpolarization of \( I_h \) activation. The reduction of \( I_h \) had been reported in spinal motoneurons in previous studies (Kjaerulff and Kiehn 2001; Takahashi and Berger 1990), but it was attributed to a time-dependent rundown of 5-HT effect on \( I_h \). Different from this rundown effect, however, the 5-HT-induced reduction of \( I_h \) observed in the present study was detectable within 3–5 min right after bath administration of 5-HT (15–20 \( \mu \)M), and more importantly, a full or partial recovery of \( I_h \) from the reduction could be obtained after 15–30 min washout. In fact, the 5-HT-induced reduction of \( I_h \) had been reported in previous studies in rat tegmental area dopamine neurons (Liu et al. 2003) and rat cerebella Purkinje neurons (Li et al. 1993; Williams et al. 2002). But this reduction of \( I_h \) was shown to be 5-HT concentration dependent (Liu et al. 2003) and could be induced only with high concentration of 5-HT (50–500 \( \mu \)M). In this study, however, a 15–20 \( \mu \)M 5-HT was enough to induce a decrease in \( I_h \) in EGFP+ neurons while the same amount of 5-HT could also induce an increase in \( I_h \) in some other EGFP+ neurons.

This unique property of \( I_h \) has never been reported in spinal neurons, and the present study is the first to report this finding. The negative modulation of \( I_h \) by 5-HT could play a positive role in modulating neuronal excitability such as enhancement of bistability in Purkinje neurons (Williams et al. 2002). However, the functional role of \( I_h \) inhibition in EGFP+ neurons is unclear. A 42% reduction of \( g_{\text{max}} \) and \(~3\) mV hyperpolarization of \( V_{\text{act}} \) could produce a fair amount of changes in membrane potential and input conductance and resulted in a potential change in neuronal excitability in the EGFP+ neurons.

As \( I_h \) could be enhanced by 5-HT through activation of a subset of receptor types that are positively coupled to adenylyl cyclase activity in various types of neurons, \( I_h \) could be also inhibited through receptors negatively coupled to adenylyl cyclase activity (Pape 1996). However, the modulatory property of \( I_h \) by 5-HT could be different with cell types. In rat tegmental area dopamine neurons, the reduction of \( I_h \) was shown to be mediated by activation of 5-HT\(_3\) receptors with involvement of protein kinase C (Liu et al. 2003). In the spinal motor system, however, little information is available about this negative modulation of \( I_h \). The mechanism underlying the reduction of \( I_h \) in EGFP+ neurons remains unknown.

SIGNIFICANCE OF 5-HT MODULATION OF \( I_h \) IN EGFP+ NEURONS. Our study defined three groups of EGFP+ neurons based on their response to 5-HT. They are neurons with \( I_h \) enhanced (1st group), reduced (2nd group), and unaltered (3rd group) by 5-HT. These neurons did not show any significant difference in \( I_h \) kinetics (Table 3) nor were they different in laminar distributions (Table 4). However, their different responses to 5-HT in intrinsic membrane properties suggested that they could play a different functional role during locomotion (Zhong et al. 2006b). A number of studies have shown that \( I_h \) plays a role in setting passive membrane properties, generating rhythmic activity, and regulating synaptic transmission in different types of cells and species (Harris-Warrick 2002; Pape 1996; Robinson and Siegelbaum 2003). In rodent spinal neurons, the property of postinhibitory rebound (PIR) induced by \( I_h \) (or calcium conductance) was regarded as one of the essential mechanisms underlying the rhythm generation during locomotion (Bertrand and Cazalets 1998; Wilson et al. 2005). On the other hand, however, \( I_h \) was also shown to be a leak conductance that induced a tonic depolarization during locomotion (Butt et al. 2002; Kiehn et al. 2000). These multiple and dynamic properties of \( I_h \) could provide spinal motor system with more flexible ways to regulate the neuronal excitability and functional performance. Therefore we could expect that the first group (majority) of EGFP+ neurons increase the neuronal excitability during locomotion. The enhanced depolarizing current from \( I_h \) and the PIR property endowed by \( I_h \) could facilitate the tonic firing or rhythm generation. The second group (minority) of the neurons, however, could reduce the depolarizing drive, inhibit the PIR property, and therefore lead to a counteraction of the excitatory drives or reduction of neuronal excitability during locomotion. \( I_h \) in these neurons might play a balance or inhibitory role in rhythm generation or limit the ability of these neurons to participate in locomotion. \( I_h \) in the third group of neurons is not altered by 5-HT. These neurons could maintain a balance between the excitatory and inhibitory effects that are produced by \( I_h \) and modulated by 5-HT. Finally, we have to...
point out that the functional role of \( I_h \) in EGFP+ neurons for generation of rhythmic activity would depend not only on its modulatory properties but also its interaction with other ionic channels such as \( I_A \) (MacLean et al. 2003, 2005; McCormick and Pape 1990), low-threshold calcium channels (McCormick and Pape 1990), or persistent sodium current (Dickson et al. 2000). These multiple factors could produce a cellular environment where \( I_h \) is either a positive or negative factor for generation of locomotion, depending on the state and activity of the EGFP+ neurons during locomotion.

**Modulation of \( I_h \) by ACh**

The present study demonstrates variable effects of ACh on \( I_h \)-mediated depolarizing sag in EGFP+ neurons. In contrast to 5-HT, ACh generally produces an inhibitory effect on \( I_h \) that is consistent with our recent observation in EGFP+ neurons (Dai et al. 2009). Enhancement of sag by ACh was never observed in this study. These results suggest that activation of cholinergic receptors in EGFP+ neurons could decrease the neuronal excitability or limit the participation of these neurons in locomotion through inhibition of \( I_h \). Reduction of sag by muscarine has been reported in recent study of spinal motoneurons in the salamander (Chevallier et al. 2006). However, little is known about ACh modulation of \( I_h \) in rodent spinal neurons. The present study is the first report to demonstrate the negative modulation of \( I_h \) by ACh in locomotion related interneurons in rodent spinal cord. The functional role of this modulation remains unknown. However, a recent study shows that cholinergic activation of \( n_m \)-type muscarinic receptors increases excitability by reducing the action potential afterhyperpolarization (Miles et al. 2007). Therefore the output of the neurons with negative modulation of \( I_h \) by ACh could be regulated by multiple ionic conductances. Their functional role is dependent on the state of motor behavior with modulation from different neuromodulators.

**\( I_h \) and sag with laminar distribution**

\( I_h \) and sag were found in \(~50\%\) of the EGFP+ neurons with almost evenly distributed in lamina VII, VIII, and X (Fig. 2, C and D). A further analysis indicated that EGFP+ neurons with sag (\( n = 29 \)) were not significantly different from the EGFP+ neurons without sag (\( n = 22 \)) in resting membrane potential (71.0 \( \pm \) 9 mV for sag neurons and 67.9 \( \pm \) 8 mV for nonsag neurons, \( P = 0.09 \)), rheobase (109.1 \( \pm \) 52 pA mV for sag neurons and 89.7 \( \pm \) 54 pA for nonsag neurons, \( P = 0.10 \)), and input resistance (393.5 \( \pm \) 233 M\( \Omega \) for sag neurons and 354.4 \( \pm \) 197 M\( \Omega \) for nonsag neurons, \( P = 0.20 \)). These results suggested that \( I_h \) might not play a dominant role in regulating the passive membrane properties of EGFP+ neurons in normal condition. Our results further indicated that no significant difference was found between the properties of \( I_h \) or sag and their laminar distribution (Tables 1 and 2), suggesting that \( I_h \) might not be identified by its laminar location or kinetic properties in EGFP+ neurons in resting state. However, \( I_h \) or sag did make significant difference in response to 5-HT (Table 3) and ACh (Fig. 9C). All these results have depicted an outline of \( I_h \) in EGFP+ neurons for us: this current could be evenly expressed in most of EGFP+ neurons in spinal ventral region (lamina VII, VIII, and X) with no substantial difference in kinetics. It may not dominate the building of passive membrane properties of these neurons in resting state. However, it could distinguish itself from modulatory properties with neurotransmitters such as 5-HT and ACh and therefore make significant contribution to regulation of dynamic properties of the EGFP+ neurons in state-dependent manner during locomotion.

**Combination of cfos-EGFP transgenic mice with other techniques**

Using cfos-EGFP transgenic mice we have demonstrated the ability to identify interneurons activated by a locomotor task (Dai et al. 2009). In this study, we further demonstrate that the specific channel properties of locomotor activity-related neurons can be investigated in vitro preparation. This new method of identifying interneurons could be particularly powerful when used with other techniques such as anterograde or retrograde labeling technique (Carlin et al. 2006) for study of the commissural interneurons specifically activated by locomotor activity. In fact, it has been shown in our recent study that a portion of the EGFP+ neurons filled with intercellular dye is commissural interneurons. These anterogradely labeled neurons locate in lamina VIII and X with axon crossing over the midline and projecting to the contralateral side (unpublished data). Furthermore, some of the EGFP+ neurons in lamina IX are also shown to be motoneurons. The combination of cfos-EGFP transgenic mice with labeling technique would allow us to target some specific types of spinal neurons which play a unique functional role in generating or mediating locomotion. On the other hand, this animal model could be also used with immunohistochemical technique to verify the neurochemical types of the EGFP+ neurons. As shown in our recent studies, the EGFP+ neurons are heterogeneous population widely distributed in spinal cord. Some of them anatomically overlap with some interneurons which have been well studied in types and functional features such as the glutamatergic Hb9 interneurons (Wilson et al. 2005) and cholinergic interneurons (Miles et al. 2007; Zagoraiou et al. 2009). The combined techniques could bridge the EGFP+ neurons with these neurons and redefine some new population of interneurons with multiple genetic or neurochemical identities. In summary, the cfos-EGFP mouse model has provided a new method of selecting neurons active during locomotion. In combination with other techniques this method can be potentially useful for study of spinal neurons underlying locomotion.

**Conclusion**

Using cfos-EGFP transgenic mice we are able to study \( I_h \) in spinal interneurons that are activated during locomotion. \( I_h \) is widely expressed in spinal ventral EGFP+ neurons. Activation of serotoninergic and cholinergic receptors in EGFP+ neurons modulates \( I_h \). The high degree of expression of \( I_h \) in EGFP+ neurons and varying effects of 5-HT and ACh on it indicate that this current plays multiple roles in initiating or generating locomotion. The finding of 5-HT and ACh-induced reduction of \( I_h \) in EGFP+ neurons suggests a novel mechanism that motor system could use to limit the participation of certain neurons in locomotion.
APPENDIX

Does acute swim stress induce EGFP expression in spinal neurons?

As discussed in our previous paper (Dai et al. 2009), in addition to locomotor task cfos expression can be induced in spinal neurons by many other factors including cell death (apoptosis), surgical procedures (noxious stimulation), uncontrollable behavior (background activity), and so on. Here we focus on another possible factor: swim stress. This issue arose from the concern that the swimming-induced locomotor activity in cfos-EGFP mice could also induce stress in these animals, and swim stress has been shown to induce cfos expression in dorsal raphe nucleus (Roche et al. 2003). It is unclear, however, if swim stress affects the cfos expression in spinal neurons, especially the ventral neurons from T12 to L4 that we have targeted for patch-clamp recording in our studies.

According to the studies by Valentino’s group, swim stress engages neuropeptide corticotropin-releasing factor inputs to GABA neurons in the dorsolateral dorsal raphe nucleus that function to inhibit 5-HT neurons and 5-HT release in the forebrain (Roche et al. 2003). This result suggests that swim stress might reduce the 5-HT release in spinal neurons which are innervated by dorsal raphe nucleus. If this is true, we would expect less c-fos expression in spinal ventral neurons since the decrease in 5-HT release might reduce the excitability of these neurons and thus reduce the cfos expression. In fact, it has been demonstrated in our recent study that 5-HT plus other neurotransmitters (N-methyl-D-aspartate, dopamine, and ACh) largely increased the number of EGFP+ neurons in spinal cord (see Fig. 2 in Dai et al. 2009). In contrary, therefore a reduction of 5-HT release should reduce EGFP expression in spinal neurons. In other words, the number of EGFP+ neurons induced by swimming task should be less than the number of EGFP+ neurons induced by walking task. As indicated in our previous study (Dai et al. 2009), however, there was no overt difference in EGFP expression between the walking and swimming slices, suggesting that the effect of swim stress (if any) on EGFP expression in spinal neurons might not be essential or distinguishable with respect to our swimming protocol. This conclusion was supported by the observation that the expression of EGFP+ neurons in cfos-EGFP mice generally agreed with the expression of cfos-neurons induced by electrical stimulation of the mesencephalic locomotor region or spontaneous treadmill locomotion in decerebrate cats where the stress activity was not involved (Dai et al. 2005b; Noga et al. 2009).

To address this issue convincingly, we did some extra experiments for swim stress test. The cfos-EGFP transgenic mice of postnatal day 8 were used for this test. Animals at this age are within the category of P6-12 that we used for this study. The animals were divided into three groups: the first group was held in housed box for 70 min as control before the surgery for slices. The second group was induced for 70 min swimming with protocol described in METHODS. The third group was used for swim stress test. The animal in this group was handled with the same procedure as the second group but only brief swimming was induced for stress test. The animal was dipped in water (26–30°C) for ~10 s and then withdrew from the water for ~5 s. The animal was then dipped in the water for other 10 s and withdrew again. This process was repeated for ~1 min (5 times). Then the animal was dried with soft tissue and placed back to the homed box. After 5-min rest, the second round of stress test was undertaken in the same animal with the same procedure. The test was repeated for eight rounds. The animal was then held in the box for 20-min rest and surgery was operated for slice preparation (METHODS). The total time of stress swimming was ~10 min and the entire procedure of the test (including the last 20 min for rest) was ~70 min. Slices of control, swimming and swim stress mice were cut at 200 µm and fixed for 20 min with 4% paraformaldehyde. They were then mounted on slides, allowed to air dry, and coverslipped with Vectashield mounting medium (Vector Laboratories H-1000). Slides were scanned and photographed with confocal image system (Olympus Fluoview 2.1).

Slices from control (left), swim stress (middle), and swimming mice (right) are shown in Fig. A1. Similar to the observation in our previous study (Dai et al. 2009), only a few of weakly labeled cells (dorsal area) are shown in control and stress slices but EGFP+ neurons are dramatically increased in swimming slice. More importantly, no substantial difference in EGFP expression can be found between the control and swim stress slices. This test rules out the short-term (~10 min), acute swim stress as a potential factor for inducing cfos-EGFP expression in spinal neurons of neonatal cfos-EGFP transgenic mice during swimming.

ACKNOWLEDGMENTS

The authors thank Dr. Robert Brownstone for valuable comments on the early work of this study and C. Gibbs, J. McVagh, S. Deschamps, G. Detillieux, M. Ellis, M. Setterbom, and E. Henson for expert technical support.

GRANTS

This study is supported by a Canadian Institutes of Health Research grant to L. M. Jordan.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
REFERENCES


