Electrophysiological and Pharmacological Properties of Locomotor Activity-Related Neurons in cfos-EGFP Mice

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INTRODUCTION

It is well established that the basic rhythm and pattern of locomotion is generated by a system of spinal neurons that make up the central pattern generator (CPG) for locomotion (Brown 1911; Grillner 1981). Numerous studies have been undertaken to identify neurons that make up the CPG. It was initially suggested that the rat CPG is anatomically localized to the L1/L2 spinal segments (Cazalets et al. 1995, 1996), but subsequent studies suggest that the hindlimb network is distributed throughout the lumbar and supralumbar spinal cord (Cina and Hochman 2000; Cowley and Schmidt 1997; Kjaerulf et al. 1994; Kremer and Lev-Tov 1997; Nishimaru and Kudo 2000; Nishimaru et al. 2000; Palmer et al. 2001). Early efforts to examine the properties of rodent neurons active during locomotion included intracellular and blind patch recordings (Kiehn et al. 1996; MacLean et al. 1995), tetrode recordings (Tresch and Kiehn 1999), and calcium imaging (Nakayama et al. 2002) from the isolated neonatal rat spinal cord.

Recently techniques have become available to identify specific interneurons in reduced spinal cord preparations such that their physiological and pharmacological properties can be examined. For example, it has been shown that the activity of ascending commissural cells can be modulated by the neurotransmitters serotonin (5-hydroxytryptamine, 5-HT) and acetylcholine (ACh) (Carlin et al. 2006; Zhong et al. 2006a). Similarly, descending commissural cells are excited by 5-HT and are rhythmically active during fictive locomotion in vitro (Zhong et al. 2006b). Populations of ventral interneurons, including EphA4 (Butt et al. 2005; Kullander et al. 2003), Dbx1 (Lanuza et al. 2004), En1 (Gosgnach et al. 2006), Chx10 (Crone et al. 2008), and Sim (Zhang et al. 2008) expressing neurons have been shown to have discrete roles in locomotion. HB9-expressing interneurons may also play a role in rhythm generation (Brownstone and Wilson 2008; Hinckley et al. 2005; Wilson et al. 2005). A discrete group of cholinergic medial partition neurons located near the central canal appears to terminate on motoneurons and control their excitability during locomotor activity by reducing the action potential hyperpolarization (Miles et al. 2007). Some of these genetically defined neurons can now be visualized in spinal cord slices through the use of fluorescent reporter genes. Nevertheless, few of these neurons have been extensively characterized in terms of their electrophysiological properties and their response to neurotransmitters that produce fictive locomotion for instance.

These efforts to define locomotor neurons in the spinal cord differ from the present study by virtue of the fact that they employed anatomical and genetic features for identification of the neurons. In contrast we use a marker of neural activity—enhanced green fluorescent protein (EGFP) expression driven by the c-fos promoter—to identify neurons active during a locomotor task in mice. In a recent study (Dai et al. 2005b), we have demonstrated that locomotion induces c-fos expression in decerebrate cat spinal neurons. Here we use a degradable form of EGFP to minimize any labeling from activity prior to a locomotor task and then target these neurons for study in spinal cord slices. We characterize the electrophysiological properties...
of EGFP-positive neurons (“locomotor neurons”) in lamina VII, VIII, and X of T12-L4 segments using whole cell patch-clamp techniques. Previous studies demonstrated that 5-HT is necessary and ACh is sufficient to induce locomotor-like activity in isolated spinal cords of neonatal rats and mice (Cowley and Schmidt 1994; Hochman et al. 1994a; Jiang et al. 1999; Kiehn and Kjaerulff 1996; MacLean et al. 1995; Smith and Feldman 1986, 1987; Smith et al. 1988). Further studies showed that several types of 5-HT receptors (5-HT1, 5-HT2, and 5-HT7) are essential in inducing locomotor-like movements in neonatal rat and mouse spinal cord (Beato and Nistri 1998; Bracci et al. 1998; Cazalets et al. 1992, 1995; Hochman et al. 2001; Lui and Jordan 2005; Madriaga et al. 2004) and in adult rat and mouse transected spinal cord (Landry and Guertin 2004; Landry et al. 2006; Ung et al. 2008). We therefore examined the modulation of intrinsic membrane properties of the EGFP+ neurons by these two neuromodulators.

We demonstrate that activation of neurons from the cfos-EGFP mice results in robust expression of EGFP sufficient for identification of these neurons and report heterogeneous anatomical and electrophysiological properties and responses to 5-HT and ACh. Preliminary reports of some of this work have been published (Brownstone et al. 2002; Dai et al. 2004).

**Methods**

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

**Production of the cfos-EGFP transgenic mice**

C-FOS PROMOTER-PD2EGFP-1. The cfos-EGFP mouse line was produced in the McMahon lab in the following manner: plasmid p301-602 (Gilman et al. 1986) was cut with HindIII and ligated to an EcoRI adaptor. The plasmid was then cut with BamHI to release a 700-bp EcoRI/HindIII-BamHI fragment that encompassed the mouse c-fos promoter (Accession No. V00727, nucleotides 1–600 plus about 100 unsequenced base pairs upstream), including the following response elements: CRE, AP1, SIE, SRE. The gel-purified EcoRI/HindIII-BamHI fragment was cut with EcoRI and ligated into EcoRI-BamHI cut pd2EGFP-1 vector. DHS' alpha subcloning efficiency (Gibco) cells were transfected with the c-fos promoter-EGFP plasmid following manufacturer’s instructions (calcium phosphate). The c-fos promoter-EGFP plasmid was purified (Invitrogen, SNAP Midi Prep), cut with EcoRI and AflIII, releasing a 1.7-kb base-pair fragment that was subsequently gel purified. Twenty micrograms of EcoRI-AflIII fragment was purified by cesium chloride gradient without ethidium bromide and then extensively dialyzed against TEN buffer (1 l, 6 times) at 4°C.

Transgenic mice were generated by the University of Kentucky Transgenic Mouse Facility. Transgene DNA was microinjected (5 ng/µl) into the pronuclei of fertilized B6C3F1 hybrid mouse embryos (Harlan) and implanted into pseudopregnant females. Mice were screened for the transgene both by dot-blot analysis and PCR of genomic DNA isolated from the tail at the time of weaning. A homozygous transgenic line was then bred.

**Dorsal root ganglion neuron (DRG) dissociation**

DRG were harvested from postnatal (P4–P13) mice. Harvesting of these cells used the same surgical procedure as described previously to harvest the spinal cord (Carlin et al. 2000). Once the spinal cord was removed from the spinal column, fine forceps were used to pull the ganglia from between the vertebrae. This first part of the procedure was performed in ice-cold dissecting artificial cerebrospinal fluid (ACSF). Once removed from the animal, the ganglia were placed in a conical tube containing room-temperature HEPES-based ACSF solution with 2.5% trypsin (Invitrogen, Carlsbad, CA). The trypsin was allowed 20 min to work before the digested tissue was rinsed (×3) with fresh HEPES ACSF containing bovine serum albumin (Sigma). The tissue was then triturated with a plastic pipette and plated directly into the glass-bottomed recording chamber. HEPES-based ACSF contained (in mM) 140 NaCl, 3 KCl, 10 HEPES, 2 MgCl2, 10 glucose, and 2 CaCl2, pH = 7.4.

**Imaging of acutely dissociated DRG**

Fluorescent images were captured using a Hamamatsu image capture and processing package (Hamamatsu, Bridgewater, NJ). This included a C2400 CCD camera, a camera controller, ARGUS 20 image processor, and HPS frame grabber and software. The images were acquired at regular intervals of 1–2 min. Frames (512) were accumulated with the ARGUS software, and images were stored on a Pentium class computer. The fluorescent light shutter was closed when not acquiring images. Using control cells alone or unstimulated cells in the field of view (n = 4; see Results) the illumination process itself did not cause any detectable increase in fluorescence expression. This is in contrast to the use of a long-pass filter (XF-02; Omega Optical), which caused an increase in GFP expression in proportion to the rate of illumination (data not shown). This latter increase in fluorescence signal is likely due to UV stimulated c-fos expression. Intensity measurements were preformed on stored images with the ARGUS software. Intensity difference was calculated using defined areas in the cell of interest and a reference cell or background.

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

Experiments were carried out on neonatal (P4-15) cfos-EGFP mice. A locomotor task (walking or swimming) was induced in the animals prior to preparation of slices. The walking protocol was used for only older mice (>P6). With this protocol, a small amount of mother mouse’s fur was used to attract the animals. The fur was put into a microcentrifuge chamber. The chamber was then placed near the nose of the animals and moved slowly to attract the animals for walking. The swimming protocol was designed for younger mice of P4–P8. Animals at this age were usually tiny in size and physically weak for long walking (60–90 min) with body weight. Therefore this protocol was used to induce locomotor task in >90% of the animals (n = 64: 44 for patch-clamp experiments and 20 for confocal images) in this study. A length of 1-cm-wide paper tape is prepared for suspending the animal by making the portion of tape that touches the animal nonadhesive with a 4-cm-long piece of the same tape placed sticky side to sticky side. This tape is then placed around the animal’s thorax, and the distal ends are 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 allows the mouse full range of motion in all limbs as well as normal breathing. The ends of the tape are placed in a clamp attached to a calibrated microdrive. The water bath is held at 26–30°C (usually 28°C) using a hotplate and thermometer. The microdrive is used to lower the mouse into the water bath. The clamp is situated on the tape in such a manner as to assure that the head is maintained above the surface of the water at all times. On being positioned with its limbs in the water, the mouse immediately begins swimming. During the first 5–10 min, the mouse is allowed to habituate to the water bath. During this time, the animal may attempt to escape the water. If this occurs, the experimenter lifts the animal out of the water for a few seconds and then places it back in. This is repeated a few times until the mouse becomes used to the water and ceases all signs of struggling. At this point, the animal begins to swim.
steadily for as long as its limbs are kept below the surface of the water. This “swimming task” continues for 60–90 min. Maintaining the temperature within the proper range is a key element to the success of the task. If the water bath is too warm, the mouse may fall asleep. If it is too cold, the animal will struggle. The duration of the task must be ≥60 min to assure that the locomotor activity is sufficient to induce c-fos expression in the locomotor neurons of the spinal cord and consequent expression of the EGFP protein in these neurons. The EGFP allows the locomotor neurons activated by the task to be visualized later in spinal cord slices taken from this mouse. The reason for using swimming task is that this new task is much more efficient in inducing locomotion-like movement in young animals than the walking task. No overt difference in expression of EGFP+ neurons was found between the walking and swimming slices.

The procedure for the preparation of spinal cord slices was as previously reported (Carlin et al. 2000). Transverse slices were cut at thickness of 180–250 μm from T12 to L4 segments and remained in the recovering ACSF for ≥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% O2-5%CO2. The EGFP-positive neurons were identified at ×40 magnification using epifluorescence with a narrow band GFP cube (Omega XF-37). The neurons were then visualized using infrared illumination and images collected (Hamamatsu camera controller C2400 and ARGUS image processor). The visualized neurons were patched (electrode resistances: 3–6 MΩ). A MultiClamp 700A patch-clamp amplifier, Digidata 1322A A/D converter, Minidigi 1A, and pClamp (9.0) software (all from Molecular Devices) were used for data acquisition. Whole cell patch recordings were made in current-clamp mode with bridge balance and capacitance compensation and in voltage-clamp mode with series resistance compensation by 70–80%. A constant hyperpolarizing current from 0 to 100 pA was injected into some of the cells to maintain the resting membrane potential at about −60 mV or lower during the recordings. The recording protocols were repeated two to three times in each condition (control, drugs, and washout etc.) with a 2–3 min interval. The reported df was measured from the time of AHP peak to the time of half AHP amplitude.

The present study is based on the experimental results from Carlin et al. (2000). Transverse slices were cut at thickness of 180–250 μm from T12 to L4 segments and remained in the recovering ACSF for ≥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% O2-5%CO2. The EGFP-positive neurons were identified at ×40 magnification using epifluorescence with a narrow band GFP cube (Omega XF-37). The neurons were then visualized using infrared illumination and images collected (Hamamatsu camera controller C2400 and ARGUS image processor). The visualized neurons were patched (electrode resistances: 3–6 MΩ). A MultiClamp 700A patch-clamp amplifier, Digidata 1322A A/D converter, Minidigi 1A, and pClamp (9.0) software (all from Molecular Devices) were used for data acquisition. Whole cell patch recordings were made in current-clamp mode with bridge balance and capacitance compensation and in voltage-clamp mode with series resistance compensation by 70–80%. A constant hyperpolarizing current from 0 to 100 pA was injected into some of the cells to maintain the resting membrane potential at about −60 mV or lower during the recordings. The recording protocols were repeated two to three times in each condition (control, drugs, and washout etc.) with a 2–3 min interval. The reported df was measured from the time of AHP peak to the time of half AHP amplitude.

**Measurement of cell membrane properties**

The membrane properties measured and calculated in this study include current threshold ($I_{th}$), voltage threshold ($V_{th}$), resting membrane potential ($E_{m}$), input resistance ($R_{m}$), membrane time constant ($τ_m$), whole cell capacitance ($C_m$), slope of frequency/current ($F-I$) relation, action potential (AP) height and width, and afterhyperpolarization (AHP) depth, and half-decay time. The $I_{th}$ was determined by step currents with 0.5-s duration and 50 pA for each step. The minimum step that could evoke a single spike or repetitive firing was taken as the $I_{th}$. The $V_{th}$ was defined as the membrane potential at which the rising rate of dV/dt > 10 mV/ms. The $V_{th}$ reported in this paper was measured from the first spike of firings evoked by a 2-s triangular current with amplitude of 0.5–1.5 nA. The resting membrane potential was monitored throughout the recordings using Axon Minidigi 1A. The reported $E_{m}$ in this paper was calculated from the membrane potentials averaged over 100 ms prior to the step currents used for determining the $I_{th}$. The measurements of AP and AHP properties were based on an averaged spike from the first one to five spikes evoked by $I_{th}$. The $V_{th}$ was used as reference value to measure the AP height and width and AHP depth. The half-decay time of AHP was measured from the time of AHP peak to the time of half AHP decaying from the peak. Step currents with duration of 2 s and step of 50 pA were injected into the cells to determine the $F-I$ relations. The instantaneous firing frequency was calculated as the reciprocal of the interspike interval (ISI) for the first, second, and steady-state (ss) spikes. The ss firing frequency was calculated as a mean rate of firings over the last 1 s duration of the firings in the tonic firing neurons (type 3) or over the last 5–10 spikes in the neurons with phasic firing (type 2). In a few of the type 2 cells that fired only a few spikes, the averaged rate of firing for all spikes was taken as the ss firing frequency. A linear regression was applied to the $F-I$ relations for calculation of the slopes. The slopes calculated from the $F-I$ relations of the first, second, and ss spikes were defined as slope 1, slope 2, and slope SS, respectively.

A 1-s hyperpolarizing step current with step of −20 to −50 pA was injected into the cells. The voltage deflection with no or minimum activation of h-current was averaged over the first 0.5 s starting from the peak deflection of the membrane potential. The $R_{m}$ was calculated by the mean value of the voltage divided by the amplitude of the corresponding step current. The time constants ($τ_m$ and $τ_f$) were determined by fitting a double decaying exponential function with form of $Y = y_0 + A_1 * \exp(V/τ_m) + A_2 * \exp(V/τ_f)$ to the averaged voltage responses to a train of five pulses (0.5 ms, −1 nA, 250-ms interval). The whole cell capacitance was calculated by the formula: $C_m = [τ_m / (R_m * τ_m + R_m * Tanh(L))]$, where $L = π(τ_m / τ_f - 1)^{1/2}$. In addition to the measurement of the preceding membrane properties, persistent inward current (PIC) and hyperpolarization activation inward current ($I_h$) were also tested in the present study. The PIC was recorded in voltage clamp by applying a slow voltage bi-ramp with duration of 5–10 s, peak of 0–30 mV, and holding potential of −70 mV (Lee and Heckman 2001). $I_h$ was recorded in current clamp with the same protocol for $R_{m}$ recording.

**Solutions and chemicals**

The dissecting ACSF contained (in mM) 25 NaCl, 188 sucrose, 1.9 KCl, 1.2 NaHPO4, 10 MgSO4, 26 NaHCO3, 1.5 kynurenic acid, and 25 glucose. The recovering ACSF contained (in mM) 118 NaCl, 4.5 KCl, 1.2 NaHPO4, 10 MgSO4, 26 NaHCO3, 1.0 CaCl2, 1.5 kynurenic acid, and 25 glucose. The recording ACSF contained (in mM) 130 NaCl, 4.5 KCl, 1.25 NaHPO4, 1.25 MgCl2, 26 NaHCO3, 2.5 CaCl2, and 10 glucose. The pH of these solutions was ~7.4 when bubbled with 95% O2-5%CO2.

The intracellular solution contained (in mM) 150 KMeSO4, 10 NaCl, 10 HEPES, 0.1 EGTA, 3 Mg-ATP, and 0.3 GTP. Alexa 594 (~3%; Invitrogen Canada) or Lucifer yellow (~1%) was added to the intracellular solution in some experiments to study the morphology of the recorded neurons. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

**RESULTS**

The present study is based on the experimental results from intracellular recordings of 72 spinal neurons (60 EGFP+ and...
EGFP expression in acutely dissociated DRG cells

The activity-dependent nature of the c-fos-driven EGFP expression was confirmed using acutely dissociated dorsal root ganglion neurons. These neurons were chosen because of their high degree of viability during the dissociation process as well as a previous report of c-fos expression in these cells due to trains of action potentials (Fields and Nelson 1994). Once dissociated, these cells were patch clamped and repeatedly depolarized under voltage-clamp conditions ($V_h = -60$ mV, test pulse to 0 mV $\times 500$ ms $\times 0.2$ Hz) while fluorescence intensity measurements were taken at regular intervals. Using this procedure, stimulated cells were seen to fluoresce more intensely during stimulation ($n = 5/6$). In a number of experiments, multiple cells were included in the field of view, and the difference of intensities was recorded for stimulated and unstimulated cells. As illustrated in Fig. 1 (A–D), the fluorescence intensity of the stimulated cell can be seen to increase dramatically, while it is unchanged in the unstimulated cell. The accompanying graph (Fig. 1E) demonstrates that the fluorescence increase corresponds to the period of stimulation. After a short delay, the intensity decreased with cessation of fluorescence increase corresponds to the period of stimulation, which demonstrates that the difference of intensities was recorded for stimulated and unstimulated cells. As illustrated in Fig. 1 (B–D), the fluorescence intensity of the stimulated cell reached a peak intensity even after termination of the stimulation. The rapidity of these fluorescence changes is consistent with the expression of a fos-β-galactosidase fusion protein that showed increased expression in as little as 15 min poststimulation in transfected neuroblastoma cells (Schilling et al. 1991). The decay of fluorescence intensity was slow and could continue for hours before returning to baseline. These experiments demonstrate that neurons from the c-fos-EGFP animals used in this study express EGFP under conditions that should increase the expression of c-fos.

EGFP expression in spinal cord slices in vitro

Previous studies have shown that certain neurotransmitters applied directly to the isolated spinal cord of the neonatal rat or mouse can induce locomotor-like activity (see Kiehn and Butt 2003 for review). To determine whether EGFP expression could be evoked in vitro by these same agents in slices from the c-fos-EGFP transgenic mice, we applied a mixture of 30 μM 5-HT, NMDA, dopamine, and ACh to slices of 150 μm thickness taken from the T12–L4 segments of P8 cfos-EGFP transgenic mice. After 2 h, control and drug-treated slices were fixed and examined using a laser scanning confocal microscope. The number and intensity of EGFP+ cells were dramatically increased in the drug-treated slices. The intensity of the EGFP+ neurons was increased by the drug cocktail to a degree that necessitated that the sensitivity (gain) of the camera had to be reduced by 20–30% of control to avoid saturation of the images. As Fig. 2 shows qualitatively, the drug cocktail induced a dramatic increase in the EGFP detectable in neurons in most laminae of the spinal cord. A pronounced increase in EGFP expression occurred in the dorsal horn, in lamina VII, VIII, and IX, and around the central canal (lamina X). Motoneurons as well as interneurons displayed increased fluorescence, showing that many cells in the presumed locomotor areas of the spinal cord including the ventromedial region (see Kiehn and Jägerulf 1998; Jägerulf and Kiehn 1996) are activated during the chemical stimulus and that these cells can be detected with the EGFP reporter. These experiments demonstrate that the c-fos-EGFP mouse model can be used to reveal neurons responsive to bath application of drugs in slice preparations.

In vivo locomotor activity induces EGFP expression

To determine whether these animals could be used to study neurons activated following locomotion, locomotor activity (swimming) was induced in intact cfos-EGFP transgenic mice.
for a period of 60–90 min. Slices prepared and fixed from these animals were compared with slices from control littermates. Figure 3 shows confocal images from control (left) and locomotion (right) groups from P4 (top), P6 (middle), and P8 (bottom) mice. In the ventral horn, numerous cells were labeled with EGFP in lamina VII, VIII, and X in the locomotion group, whereas only a few cells were labeled in the same region in the control group. Thus it is clear that the swimming task induced EGFP expression in neurons in the locomotor area of the ventral horn. In this study, lamina VII, VIII and X were set as target areas from which the EGFP+ neurons were selected for patch-clamp recordings.

EGFP-positive cells were also seen in dorsal horn areas in both control and locomotion groups. This is not surprising, as dorsal horn neurons are expected to be activated and express Fos during both the locomotor task (Dai et al. 2005b) and the surgical procedures themselves (Coggeshall 2005). Neurons in the dorsal horn were not targeted in this study, but it is striking that a group of neurons in the medial part of the dorsal horn (laminae I–VI) was activated due to the locomotor task. Recent observations demonstrate rhythmically active neurons in this region during locomotor activity (J. M. Wilson, E. Blagovechtchenki, and R. M. Brownstone, unpublished data). Figure 3 also shows that the EGFP-labeled cells are reduced with age in both control and locomotion groups. However, the labeled cells appear constantly with less correlation to the animal ages compared with the control ones. The dramatic increase in EGFP-labeled cells in locomotion groups validates this animal model for the present study.

Electrophysiological properties of EGFP-positive neurons in the ventral horn

The results presented in this study were based on the data collected from 55 neurons sampled in laminae VII, VIII, and X. Of the 55 neurons, 43 neurons were EGFP positive and 12 were non-EGFP positive. The 55 neurons were selected for data analysis based on the criteria that resting $E_m \approx (or >) -60$ mV, AP overshoot $> (or \approx) 0$ mV, and $R_{in} > 150$ M$\Omega$.

An example of an EGFP-positive neuron patched in the target area is shown in Fig. 4A. The fluorescence image of EGFP could be visualized on the monitor (Fig. 4A1). The same cell is shown in an infrared image (Fig. 4A2, arrow) and patched with a glass pipette electrode (A3). This cell was located in lamina VIII as shown in a low-power ($\times$10) infrared image (Fig. 4A4). Figure 4B shows an EGFP+ neuron filled with Lucifer yellow after intracellular recordings. The image displays the detailed morphology of the neuron (round soma with $>4$ stem dendrites). Figure 4C is an illustration of the laminar distribution of 39 EGFP+ neurons patched in the target area for which the low-power images of the electrode placement were available. Cells located in the left side of the slice were mirrored onto right side.

Three types of EGFP+ neurons

The EGFP+ neurons in lamina VII, VIII, and X could be classified into three types based on their firing patterns in response to depolarizing step currents: single spike (type 1), phasic firing (type 2), and tonic firing (type 3). The similar firing pattern has been reported in rat spinal neurons in ventral horn (Szucs et al. 2003; Theiss and Heckman 2005; Theiss et al. 2007), superficial horn (Prescott and De Koninck 2002), and deep dorsal horn (Hochman et al. 1997). In response to a depolarizing step current, neurons of type 1 elicited only one to two spikes (Fig. 5A1); the type 2 fired briefly with firing duration $<1$ s (B1); the type 3 fired repetitively for a few or tens of seconds (C1). In addition to differences in firing patterns, the three types of EGFP+ neurons also showed differences in some electrophysiological properties, such as current threshold and input resistance (Fig. 5). In Fig. 5A1 a single spike was elicited by a step current of 100 pA for 2-s duration in a type 1 cell in lamina VII. The $R_{in}$ was calculated as 230 M$\Omega$ for this cell (Fig. 5A2). With half the amount of the step current (50 pA), brief firing was evoked in a type 2 cell in lamina X (Fig. 5B1), which had a $R_{in}$ of 457 M$\Omega$ (Fig. 5B2). The same amount of current produced tonic firing in a type 3 cell in lamina VIII (Fig. 5C1). The $R_{in}$ was 303 M$\Omega$ for this cell (Fig. 5C2).
Two subtypes of EGFP+ neurons were observed in our experiments based on their firing properties. One displayed a “slow” type of repetitive firing (Szucs et al. 2003) and the other a “delayed” type of phasic firing (Hochman et al. 1997; Prescott and De Koninck 2002). For simplicity, we classified the slow type ($n = 3$) as type 3 and delayed type ($n = 2$) as type 2. Table 1A summarizes the membrane properties of 55 neurons (43 EGFP+ and 12 EGFP−) sorted in laminae VII, VIII, and X. No significant difference is found between the EGFP+ and EGF− neurons in the membrane properties shown in the table. Laminar distributions of these neurons predicted differences only in input resistance and whole cell capacitance. The neurons in lamina X displayed the highest $R_{in}$ and lowest $C_m$, whereas the cells in lamina VII had the lowest $R_{in}$ and highest $C_m$. These results may suggest a graded distribution of the increased size in EGFP+ neurons from central canal (lamina X) to ventral areas (lamina VII and VIII). The majority of EGFP+ neurons in lamina VII were type 1 or type 3, whereas lamina VIII neurons tended to be type 3. Lamina X neurons were of all three types. Thus the EGFP+ neurons located in the targeted laminae did not differ dramatically in terms of membrane properties or types, but the differences in $R_{in}$ and $C_m$ suggest that a detailed analysis of cell morphology may yield new ways of classifying locomotor neurons in the ventral horn.
We also sorted the data according to the cell types to show the difference in membrane properties. Because there is no significant difference between the EGFP+/H11001 and EFGP+/H11002 neurons, we put both groups together. The results are summarized in Table 1B. In general, type 1 neurons tended to possess the smallest $R_{in}$ and AHP and the biggest $I_{th}$ and $C_m$, whereas type 2 neurons had the minimum $I_{th}$ and $C_m$ and the maximum $R_{in}$, $\tau_m$, and AP width. The type 2 neurons also showed a steeper slope of the $F-I$ relation than that of type 3 neurons. The properties of type 3 neurons generally fell between those of types 1 and 2 expect for AP and AHP, where type 3 neurons had the minimum AP width and the maximum AHP height and half decay time.

The difference in firing patterns did not strictly correspond to the difference in the neuron’s laminar location and membrane properties. The type 2 neurons were more concentrated in lamina X (73%, $n = 15$) than in other areas while neurons of types 1 and 3 appeared evenly distributed in lamina VII, VIII, and X. The functional significance of the condensed distribution of type 2 neurons is unknown. However, type 2 neurons were significantly different from type 1 in $E_m$, $I_{th}$, $R_{in}$, and AP properties, suggesting that the type 2 neurons are a
produced by a tonic firing (type 3).

TABLE 1A. Membrane properties of the EGFP+ neurons classified in laminar distribution

<table>
<thead>
<tr>
<th>Lamina VII (19)</th>
<th>Lamina VIII (13)</th>
<th>Lamina X (23)</th>
<th>Significance</th>
<th>EGFP+ (43)</th>
<th>EGFP− (12)</th>
<th>Total (55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E_{m}, mV</td>
<td>−65.5 ± 6</td>
<td>−69.9 ± 6</td>
<td>−69.7 ± 8</td>
<td>NS</td>
<td>−68.6 ± 8</td>
<td>−72 ± 12</td>
</tr>
<tr>
<td>I_{th}, pA</td>
<td>89.5 ± 51</td>
<td>111.5 ± 58</td>
<td>91.3 ± 49</td>
<td>NS</td>
<td>98.7 ± 56</td>
<td>87.5 ± 31</td>
</tr>
<tr>
<td>R_{m} (MΩ)</td>
<td>283.5 ± 124</td>
<td>321.6 ± 141</td>
<td>480.6 ± 252</td>
<td>*</td>
<td>378.3 ± 217</td>
<td>356.8 ± 171</td>
</tr>
<tr>
<td>τ_{m}, ms</td>
<td>21.0 ± 16</td>
<td>19.7 ± 9</td>
<td>24.8 ± 13</td>
<td>NS</td>
<td>20.2 ± 12</td>
<td>28.7 ± 13</td>
</tr>
<tr>
<td>C_{m}, pF</td>
<td>4.7 ± 2</td>
<td>4.4 ± 2</td>
<td>3.8 ± 2</td>
<td>NS</td>
<td>4.1 ± 2</td>
<td>4.6 ± 2</td>
</tr>
<tr>
<td>V_{th}, mV</td>
<td>−45.8 ± 11</td>
<td>−38.5 ± 10</td>
<td>−37.3 ± 11</td>
<td>NS</td>
<td>−39.5 ± 8</td>
<td>−42.9 ± 17</td>
</tr>
<tr>
<td>AP height, mV</td>
<td>49.8 ± 14</td>
<td>42.6 ± 11</td>
<td>49.1 ± 11</td>
<td>NS</td>
<td>46.2 ± 13</td>
<td>53.1 ± 11</td>
</tr>
<tr>
<td>AP width, ms</td>
<td>3.6 ± 1</td>
<td>3.2 ± 1</td>
<td>4.2 ± 1</td>
<td>NS</td>
<td>3.7 ± 1</td>
<td>4.0 ± 1</td>
</tr>
<tr>
<td>AHP depth, mV</td>
<td>13.4 ± 6</td>
<td>17.3 ± 8</td>
<td>14.2 ± 6</td>
<td>NS</td>
<td>14.5 ± 6</td>
<td>14.1 ± 6</td>
</tr>
<tr>
<td>AHP 1/2 decay, ms</td>
<td>46.3 ± 34</td>
<td>47.4 ± 30</td>
<td>53.1 ± 41</td>
<td>NS</td>
<td>49.4 ± 33</td>
<td>47.2 ± 46</td>
</tr>
<tr>
<td>Slope 1, Hz/nA</td>
<td>175.9 ± 74</td>
<td>165.4 ± 168</td>
<td>204.8 ± 93</td>
<td>NS</td>
<td>189.4 ± 118</td>
<td>126.1 ± 47</td>
</tr>
<tr>
<td>Slope 2, Hz/nA</td>
<td>143.5 ± 68</td>
<td>117.8 ± 93</td>
<td>147.1 ± 55</td>
<td>NS</td>
<td>141.5 ± 72</td>
<td>101.2 ± 33</td>
</tr>
<tr>
<td>Slope SS, Hz/nA</td>
<td>122.3 ± 66</td>
<td>82.2 ± 30</td>
<td>112.8 ± 43</td>
<td>NS</td>
<td>109.8 ± 52</td>
<td>98.8 ± 28</td>
</tr>
</tbody>
</table>

Parentheses enclose n values. Values are means ± SD. EGFP, enhanced green fluorescent protein. *, significant difference with P < 0.005 performed by single-factor ANOVA for the cells’ laminar distributions. NS: no significant difference.

FIG. 5. Three types of EGFP+ neurons. The EGFP+ neurons were classified into 3 types based on their firing patterns (A1–C1, top) in response to depolarizing step currents (2 s, 50 pA for each step; A1–C1, bottom). The passive membrane responses (A2–C2, top) to hyperpolarizing step currents (1 s, −50 pA for each step; A2–C2, bottom) were used to calculate input resistance by Ohm law. A1: cells of single spike (type 1). A1: 2 step currents were injected into a type 1 cell and only one single spike was elicited by the 2nd step (2 s, 100 pA). A2: a hyperpolarizing step current (1 s, −50 pA) was injected into the same cell, and the voltage deflection was used to calculate the R_{m} (230 MΩ). B1: cells of phasic firing (type 2). B1: a 50-pA step current evoked a brief firing in a type 2 cell for −0.6 s. B2: the cell membrane potential was hyperpolarized by a −50-pA step current, and the R_{m} was calculated as 457 MΩ in this cell. C1: cells of tonic firing (type 3). C1: the tonic firing in a type 3 cell was evoked by a 50 pA depolarizing step current. C2: the hyperpolarization of membrane potential was produced by a −50-pA step current. R_{m} = 303 MΩ in this cell.

Membrane currents mediated by multiple channels

Hyperpolarization-activated inward current (I_{h}) was tested in this study. An example is shown in Fig. 6A (and Fig. 5B2). The I_{h} was activated in a type 1 neuron in lamina X by a step current of −50 pA and 1-s duration (Fig. 6A1). Some neurons also exhibited postinhibitory rebound after termination of the step current. Figure 6A2 shows that two hyperpolarizing step currents with −50 pA for each step were injected into a type 3 neuron in lamina VII. A small I_{h} was activated by the first step current with a spike elicited by the postinhibitory rebound.
Persistent inward currents (PICs) were also tested in this study. The currents could be explored by a slow voltage ramp (Lee and Heckman 2001) as shown in Fig. 6B1, where a 5-s triangular voltage ramp from −70 to 0 mV was applied to a type 3 EGFP+ neuron in lamina VIII. A small PIC (∼50 pA) with negative current slope was demonstrated on the rising phase of the ramp. A larger PIC was also observed in the descending phase of the ramp while a small PIC (∼10 pA) was measured on the rising phase (1–2 Hz) when the membrane potential was depolarized (Fig. 7A). Using voltage clamp, 5-HT induced membrane current oscillations were observed in 7 of 20 cells tested. Similar observations were reported in recent studies in ascending commissural interneurons in the neonatal mouse (Carlín et al. 2006; Zhong et al. 2006a) and in previous studies in rat spinal interneurons in lamina VII (MacLean et al. 1995) and X (Hochman et al. 1994a,b). Figure 7 shows a voltage oscillation in a type 3 neuron in lamina X after bath application of 20 μM 5-HT. The 5-HT depolarized the membrane potential by ∼10 mV (Fig. 7A1) and induced membrane potential oscillations within ~2 min of application. The peak-to-peak amplitude of these oscillations was ~7 mV and frequency ~4 Hz (Fig. 7A2). The oscillations were voltage dependent with a small increase in frequency (1–2 Hz) when the membrane potential was depolarized (Fig. 7A3) and a small decrease in the frequency (~2 Hz) when the membrane potential was hyperpolarized (A4). The amplitude of the oscillations was relatively stable when the membrane potential was perturbed by the step currents. The oscillations stopped after 15 min washout (Fig. 7A, I and 2).

Using voltage clamp, 5-HT induced membrane current oscillations were observed in another EGFP+ neuron in lamina VII (Fig. 7B1). The neuron was recorded with membrane potential clamped at ~70 mV and 2-amino-5-phosphonovaleric acid (APV, 15 μM) and 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 10 μM) applied in the bath (Fig. 7B1). Bath application of 5-HT (20 μM) induced the current oscillations with peak-to-peak amplitude of ~50 pA and frequency of ~6 Hz (Fig. 7B2). Of the seven neurons in which 5-HT induced oscillations, two were located in lamina VII, three in lamina VIII, and two in lamina X. Five of the neurons were classified as type 3, one as type 2, and one was not tested in current clamp. The mean value of peak-to-peak amplitude for the six cells recorded with current clamp was 8.6 mV from P4–P8 mice, PIC was demonstrated in 51% of the neurons (34/67), 56% of the EGFP+ neurons (31/55), and 25% of EGFP- neurons (3/12). The expression of PICs was not related to the lamina distribution of the neurons.

### 5-HT-induced membrane potential oscillations

5-HT-induced membrane potential oscillations were observed in 7 of 20 cells tested.
(from 7 to 10 mV), and the mean frequency was 8.7 Hz (from 4 to 16.4 Hz). The 5-HT-induced membrane potential oscillations appeared to be related to the neuron types (2 and 3) but not laminar distribution (n/H11005/7).

5-HT modulation of cell membrane properties

As 5-HT is critical for locomotor activity. Its effect on EGFP neuron are investigated in this study. Our study shows that 5-HT modulated membrane properties of EGFP neuron and increased neuronal excitability. A typical example is shown in Fig. 8. Repetitive firing was evoked by step currents injected into a type 3 neuron in lamina VII (Fig. 8A1). Bath application of 30 μM 5-HT generated 11 additional spikes with no change in E_m (Fig. 8A2). This increased excitability by 5-HT was accompanied by hyperpolarization of V_th by ~2.8 mV (Fig. 8A3), reduction of AHP by ~1.6 mV (A3), and increase of R_m by ~7.5 MΩ. The slope of F-I relation was reduced by 38.1 Hz/nA without shift of the F-I curve (Fig. 8B).

These data suggest that neuronal excitability is modestly increased by 5-HT especially in the lower range of injected current (<200 pA).

The effects of 5-HT on 14 neurons (11 EGFP+ and 3 EGFP−) are summarized in Table 2. Because 5-HT-induced changes in the 11 EGFP+ neurons are generally agreed with those in all 14 neurons (see Table 2), the data from the 14 neurons are used in the following presentation. Of the 14 neurons, 5 were located in lamina VII, 4 in lamina VIII, and 5 in lamina X. Three were type 1, two were type 2, and nine were type 3. The significant changes induced by 5-HT in the membrane properties included an increase in R_in (64.3 ± 90 MΩ, 18.5%), hyperpolarization of V_th (5.1 ± 6 mV), increase in AP width (0.6 ± 1 ms) and reduction in AHP depth (1.9 ± 3 mV). Reduction in membrane potential (~1.3 ± 7 mV), rheobase (~3.6 ± 31 pA), AP height (~1.7 ± 11 mV), and AHP half decay (~8.7 ± 31 ms) were also observed in these neurons but these changes were not significant.
5-HT had a paradoxical effect on membrane potential. While 5-HT depolarized the membrane potential in 50% of the recorded neurons \((n = 14)\), another 50% of the neurons displayed a hyperpolarization of membrane potential by 5-HT. We separated these neurons into two groups based on their membrane potential response to 5-HT and summarized the effects of 5-HT on their membrane properties in Table 2. The results show that although 5-HT induced a strong and opposite effect on membrane potential, changes in other membrane properties in these two groups of cells were generally the same (only small, nonsignificant difference in AHP 1/2 decay). These results suggest that the variable effects of 5-HT on membrane potential may not result from the changes in other membrane properties in these two groups of cells but from the step current injected into cells for determination of the \(I_{\text{th}}\) was increased by 50 pA at each step, therefore any changes in \(I_{\text{th}} < 50\) pA would not be detected.

Data from 8 (all type 3) of the 14 cells were used to calculate the \(F-I\) relations. Five of the eight cells showed a significant reduction in the \(F-I\) slope, accompanied by a left \((n = 1)\) or up shift \((n = 4)\) of the \(F-I\) curves. Two of the eight cells showed an increased in \(F-I\) slope with nonsift \((n = 1)\) or right shift \((n = 1)\) of the \(F-I\) curves. A decrease in neuronal output was observed in one cell that showed a down shift of the \(F-I\) curve with unchanged slope. These data demonstrate that 5-HT generally increased the neuronal excitability and/or enhanced the output of EGFP+ neurons although the effects were variable.

**ACh modulation of cell membrane properties**

Modulation of EGFP+ neurons by ACh was also investigated \((n = 19)\). A typical example is shown in Fig. 9, which demonstrates recordings from a type 3 neuron in lamina VII. Bath application of ACh (20 \(\mu\)M) depolarized the membrane potential by \(-17\) mV with increased background noise and
spontaneous firing (Fig. 9A). ACh also led to a hyperpolarization of $V_{th}$ by $\sim 4.1$ mV (Fig. 9B), increase of the AHP by $\sim 3.5$ mV (B), reduction of $R_m$ by $\sim 55 \, \Omega$ (C); and lowering of the $F-I$ slope by $\sim 35.6 \, \text{Hz/nA}$ (D). Note that the $I_h$ was also blocked by ACh in this neuron (Fig. 9C). The change in the $F-I$ relation suggests that the excitability of the cell was increased by ACh in the lower range of injected current $\leq 250 \, \text{pA}$ but reduced in the higher range over 250 pA. This “current-dependent” modulation of neuronal excitability might result from a nonlinear balance of excitatory (slow depolarization of $E_m$ and lowering of $V_{th}$) and inhibitory (increase of AHP and reduction of $R_m$ and $I_h$) forces driven by ACh.

The effects of ACh on neuronal membrane properties from 19 neurons (15 EGFP+ and 4 EGFP−) are summarized in Table 3. The table is organized in the same format as Table 2 for similar observation of changes in membrane potential with 5-HT and ACh. Because the ACh-induced changes in the 15 EGFP+ neurons are not significantly different from those in all 19 neurons (see Table 3), the data from the 19 neurons are used in the following presentation. Of 19 recorded cells, 4 were located in lamina VII (1 type 1 and 3 type 3), 4 in lamina VIII (1 type 1, 1 type 2, and 2 type 3), and 11 in lamina X (1 type 1, 5 type 2, and 5 type 3). Although $>50\%$ of the cells were distributed in lamina X and the majority of the cells were type 3, the ACh-induced changes in membrane properties were not significantly related to either laminar distribution or cell type.

The significant changes induced by ACh include a depolarization of $E_m (3.7 \pm 9 \, \text{mV})$, reduction of $R_m (56.4 \pm 78 \, \Omega)$, 13.2\%; hyperpolarization of $V_{th} (3.9 \pm 4 \, \text{mV})$, reduction of AP amplitude ($-5.5 \pm 12 \, \text{mV}$) with an increase in AP width ($0.3 \pm$)

### Table 2. 5-HT-induced changes in cell membrane properties

<table>
<thead>
<tr>
<th>Changes in Membrane Properties</th>
<th>Control</th>
<th>EGFP+</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>14</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>$E_m$, mV</td>
<td>$-72.3 \pm 8$</td>
<td>$-72.9 \pm 8$</td>
<td>$-1.3 \pm 7$</td>
</tr>
<tr>
<td>$I_{AHP}$, pA</td>
<td>107.1 ± 61</td>
<td>118.2 ± 64</td>
<td>$-3.6 \pm 31$</td>
</tr>
<tr>
<td>$R_m$, MΩ</td>
<td>347.8 ± 111</td>
<td>342.5 ± 123</td>
<td>$64.3 \pm 90^*$</td>
</tr>
<tr>
<td>$V_{th}$, mV</td>
<td>$-39.6 \pm 10$</td>
<td>$-39.7 \pm 11$</td>
<td>$-5.1 \pm 6^*$</td>
</tr>
<tr>
<td>AP height, mV</td>
<td>43.5 ± 13</td>
<td>39.2 ± 11</td>
<td>$-1.7 \pm 11$</td>
</tr>
<tr>
<td>AP width, ms</td>
<td>3.8 ± 1</td>
<td>3.9 ± 1</td>
<td>$0.6 \pm 1^*$</td>
</tr>
<tr>
<td>AHP depth, mV</td>
<td>16.4 ± 8</td>
<td>15.8 ± 8</td>
<td>$-1.9 \pm 3^*$</td>
</tr>
<tr>
<td>AHP 1/2 decay, ms</td>
<td>52.1 ± 43</td>
<td>46.3 ± 27</td>
<td>$-8.7 \pm 31$</td>
</tr>
<tr>
<td>Slope SS, Hz/nA</td>
<td>88.3 ± 37</td>
<td>88.1 ± 39</td>
<td>$-11.1 \pm 37$</td>
</tr>
</tbody>
</table>

$^\dagger$, depolarization ($\geq 2 \, \text{mV}$ in average); $^\ddagger$, hyperpolarization ($\leq -2 \, \text{mV}$ in average); $^\ast$, significant difference with $P < 0.05$ and $^*$, $P < 0.005$ performed by paired two-sample $t$-test.
ACh modulation of membrane properties of EGFP+ neuron. A: recordings were made from a type 3 cell in lamina VII. A: 20 μM ACh was applied to the bath solution, and the membrane potential was depolarized by ~17 mV after 1-min application of the drug. Spontaneous firing occurs occasionally during the depolarization of the membrane potential. B1: the hyperpolarization of Vm and increase of afterhyperpolarization (AHP) were shown in the repetitive firings evoked by a 2-s ramp current starting from 0 pA. Spike trains from control (- - -) and ACh (---) were overlapped with unchanged baseline for resting membrane potentials. Voltage thresholds were marked by dots on the spikes (black for control and open for ACh). B2: the 1st 5 spikes from both control and ACh conditions were averaged, respectively, and overlapped on each other (- - -, control; ---, ACh) to show the changes in Vm, AHP and action potential (AP) in this neuron. C: a hyperpolarizing current (1 s, -100 pA) was injected into the same cell before and after the ACh application. Membrane potential deflections from the control (- - -) and ACh (---) conditions were overlapped and aligned to the resting membrane potentials. Spikes were elicited from postinhibitory rebound in both conditions. ACh hyperpolarized the Vm by ~4.1 mV, reduced input resistance by ~55 MΩ and increased the AHP by ~3.5 mV in this neuron. D: the F-I curves were plotted for steady-state spikes. ACh reduced the F-I slope by ~35.6 Hz/nA. The excitability of the cell was increased in the lower range of injected current (~250 pA) but slightly reduced in the higher range over 250 pA.

Table 3: ACh-induced changes in cell membrane properties

<table>
<thead>
<tr>
<th>Changes in Membrane Properties</th>
<th>Total</th>
<th>EGFP+</th>
<th>Mean</th>
<th>EGFP+</th>
<th>↑ E_m Total</th>
<th>↓ E_m Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>19</td>
<td>15</td>
<td>19</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E_m, mV</td>
<td>-68.7 ± 7</td>
<td>-69.3 ± 7</td>
<td>3.7 ± 9†</td>
<td>3.1 ± 9†</td>
<td>10.4 ± 7†</td>
<td>-5.7 ± 5†</td>
</tr>
<tr>
<td>I_in, pA</td>
<td>73.7 ± 31</td>
<td>66.7 ± 24</td>
<td>2.6 ± 48</td>
<td>10.4 ± 48</td>
<td>-10.0 ± 39</td>
<td>25 ± 69</td>
</tr>
<tr>
<td>R_m, MΩ</td>
<td>426.7 ± 241</td>
<td>467.6 ± 242</td>
<td>-65.7 ± 78*</td>
<td>-69.6 ± 78‡</td>
<td>-60.9 ± 92†</td>
<td>-37.2 ± 68</td>
</tr>
<tr>
<td>V_m, mV</td>
<td>-40.7 ± 10</td>
<td>-41.8 ± 8</td>
<td>-3.9 ± 4*</td>
<td>-3.3 ± 3*</td>
<td>-3.4 ± 4‡</td>
<td>-5.6 ± 3*</td>
</tr>
<tr>
<td>AP height, mV</td>
<td>50.4 ± 13</td>
<td>50.3 ± 12</td>
<td>-5.5 ± 12†</td>
<td>-5.6 ± 12‡</td>
<td>-6.6 ± 16</td>
<td>-2.8 ± 10</td>
</tr>
<tr>
<td>AP width, ms</td>
<td>3.3 ± 1</td>
<td>3.1 ± 1</td>
<td>0.3 ± 1†</td>
<td>0.3 ± 0.7†</td>
<td>0.4 ± 1</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>AHP depth, mV</td>
<td>14.1 ± 4</td>
<td>14.0 ± 5</td>
<td>1.1 ± 3‡</td>
<td>0.8 ± 3‡</td>
<td>1.4 ± 4</td>
<td>0.5 ± 2</td>
</tr>
<tr>
<td>AHP 1/2 decay, ms</td>
<td>51.6 ± 30</td>
<td>50.7 ± 30</td>
<td>3.8 ± 15</td>
<td>3.0 ± 15</td>
<td>0.4 ± 17</td>
<td>5.9 ± 15</td>
</tr>
<tr>
<td>Slope SS, Hz/nA</td>
<td>94.8 ± 30</td>
<td>90.1 ± 29</td>
<td>4.8 ± 52</td>
<td>12.2 ± 52</td>
<td>12.5 ± 59</td>
<td>-2.4 ± 5</td>
</tr>
</tbody>
</table>

†, depolarization (~2 mV in average); ‡, hyperpolarization (~2 mV in average); †, significant difference with P < 0.05 and *, P < 0.005 performed by paired two-sample t-test.
curve. These changes in F-I relation demonstrate the ACh modulation of neuronal excitability and output through a balance of excitatory and inhibitory forces driven by ACh in the EGFP+ neurons.

Combined effects of 5-HT and ACh on EGFP+ neurons

The combined effects of 5-HT and ACh on cell membrane properties were investigated in this study to examine the relative mechanisms mediating the modulation of membrane properties by the two agents. Figure 10 shows modulation of neuronal properties by these agents with recordings made from a type 3 neuron in lamina VIII. In control, the \( R_{\text{in}} \) was \( \sim 540 \) MΩ (Fig. 10A1) and \( V_{\text{th}} \) was \( -35.0 \) mV (A2). Bath application of 20 μM ACh reduced the \( R_{\text{in}} \) by \( \sim 190 \) MΩ (Fig. 10B1) and hyperpolarized the \( V_{\text{th}} \) by 8.6 mV (B2). Subsequent application of 20 μM 5-HT increased the \( R_{\text{in}} \) by \( \sim 180 \) MΩ from 350 MΩ measured in ACh condition (Fig. 10C1) and further hyperpolarized the \( V_{\text{th}} \) by 9.1 mV from \( -43.6 \) mV measured in ACh condition (C2). 5-HT also induced excitatory postsynaptic potentials (EPSPs) in this cell (Fig. 10C1). This figure also provides evidence for a reduction of \( I_{\text{h}} \) by ACh (Fig. 10B1). Interestingly, this reduction is reversed by subsequent application of 5-HT (Fig. 10C1). A similar reduction (or blockade) of \( I_{\text{h}} \) by ACh is also shown in Fig. 9C. These results demonstrate that the effects of 5-HT and ACh on EGFP+ neurons can be additive (e.g., \( V_{\text{th}} \)) or subtractive (e.g., \( R_{\text{in}} \) and \( I_{\text{h}} \)), depending on the specific membrane properties they modulate.

Eleven cells were investigated for combined modulation of cell membrane properties by 5-HT and ACh. Six cells were recorded with application of ACh followed by 5-HT. ACh reduced the \( R_{\text{in}} \) by \( -63.5 \pm 134 \) MΩ, and subsequent application of 5-HT reversed this reduction of \( R_{\text{in}} \) and led to an increase in \( R_{\text{in}} \) by 84.3 \( \pm 147 \) MΩ. Thus prior application of ACh did not reduce the ability of 5-HT to increase \( R_{\text{in}} \), consistent with the notion that these two agents may alter \( R_{\text{in}} \) by different mechanisms. ACh hyperpolarized the \( V_{\text{th}} \) by \( -3.4 \pm 4 \) mV, whereas 5-HT further lowered it by an additional \( -4.3 \pm 5 \) mV. Thus prior application of ACh did not diminish the ability of 5-HT to hyperpolarize voltage threshold, suggesting that the two agents may act by different mechanisms to achieve the same effect on \( V_{\text{th}} \). Similarly, ACh increased AHP depth (0.1 \( \pm 2 \) mV) and width (1.1 \( \pm 0.5 \) ms), whereas 5-HT reduced this (\( -1.9 \pm 3 \) mV; \( -6.7 \pm 10 \) ms). In another five cells, the inverse sequence of drug application was used. 5-HT increased the \( R_{\text{in}} \) (24.0 \( \pm 83 \) MΩ), hyperpolarized \( V_{\text{th}} \) (\( -7.0 \pm 5 \) mV), and reduced AHP (\( -2.0 \pm 1 \) mV), whereas ACh applied subsequent to 5-HT reduced \( R_{\text{in}} \) (\( -52.4 \pm 41 \) MΩ), further hyperpolarized the \( V_{\text{th}} \) (\( -5.6 \pm 8 \) mV), and increased AHP (2.2 \( \pm 2 \) mV).

In general, the effects of 5-HT after ACh or the effects of ACh after 5-HT were consistent with those of 5-HT and ACh alone on the EGFP+ neurons as listed in Tables 2 and 3. Thus neither agent alters the modulatory properties of the other on EGFP+ neurons, consistent with the suggestion that their actions on membrane properties of these cells are exerted by separate mechanisms. The additive effects of 5-HT and ACh on \( V_{\text{th}} \) would increase neuronal excitability, whereas the subtractive effects of 5-HT and ACh on \( R_{\text{in}} \) and AHP would either increase or decrease the cell excitability depending on the balance of the forces driven by the two agents.

Changes in membrane potential with modulation of intrinsic membrane properties

Both 5-HT and ACh induced variable effects on membrane potential. Because \( E_{\text{m}} \) and \( R_{\text{in}} \) were both shown to be modulated by 5-HT (Table 2) and ACh (Table 3), we would...
expect that the changes induced by 5-HT and ACh in $E_m$ could result from the changes in $R_{in}$. Figure 11 shows the correlations between $E_m$ and $R_{in}$ and the changes induced by 5-HT ($n = 14$) and ACh ($n = 19$). No significant correlation was found between $E_m$ and $R_{in}$ (Fig. 11, A1 and B1) before or after administration of 5-HT (correlation coefficient $R = 0.24$ for control, 0.40 for 5-HT) or ACh ($R = 0.11$ for control, 0.43 for ACh). These results suggested that the resting membrane potential was not regulated simply by the passive membrane properties such as the size of EGFP+ neurons. Also, the $\Delta E_m$ was not correlated to the $\Delta R_{in}$ in either 5-HT ($R = 0.05$, Fig. 11A2) or ACh ($R = 0.15$, Fig. 11B2) condition. Furthermore, the changes in $E_m$ and $R_{in}$ were neither correlated to the laminar distributions of the neurons nor to the types of the neurons (not shown). These results suggest that modulation of some unknown active (rather than passive) membrane properties by 5-HT and ACh result in the observed changes in membrane potentials in EGFP+ neurons.

**DISCUSSION**

In this study, we demonstrate a new method by which neurons active in locomotion can be identified and their intrinsic properties studied in the mouse spinal cord. Identification of spinal interneurons involved in locomotion is a necessary step to understand how the spinal cord produces rhythm and pattern. As shown in other systems, such as the stomatogastric ganglion (Marder and Bucher 2001), it is necessary to understand neuronal connections, their intrinsic properties, and the modulation of these properties to understand rhythm generation. In the adult cat, interneurons can be identified by their location and synaptology (Jankowska 2008). However, the intrinsic and modulatable properties of these neurons have not been studied. In the mouse, interneurons can be identified through labeling with GFP driven by, for example, transcription factors expressed during development (e.g., Wilson et al. 2005; Zhang et al. 2008), or through retrograde labeling with dyes (Carlin et al. 2006; Eide et al. 1999; Stokke et al. 2002).

With these techniques, the intrinsic membrane properties of the identified neurons can be studied directly in either in vitro or in vivo preparations. The present study demonstrates a method of studying spinal interneurons that are functionally activated by locomotor activities and visualized by EGFP expression driven by the promoter for the immediate early gene, c-fos. The membrane properties of the EGFP+ neurons and their modulatory properties by neurotransmitters 5-HT and ACh are studied in detail in this study. A conceptually similar circadian clock gene promoter-driven d2EGFP transgenic reporter mouse line has been used to target and study functionally activated biological clock neurons and circadian modulation of locomotor behavior (Ciarleglio et al. 2009; Kuhlman et al. 2003; Quintero et al. 2003).

**A** Correlation between $E_m$ and $R_{in}$ and changes induced by 5-HT

**B** Correlation between $E_m$ and $R_{in}$ and changes induced by ACh

**FIG. 11.** Correlation analysis between membrane potential and input resistant. A: correlations between $E_m$ and $R_{in}$ in both control ($\circ$) and 5-HT (●) conditions are established from 14 cells in A1. Changes induced by 5-HT in $E_m$ and $R_{in}$ in the same group of neurons are plotted in A2. B: similar to A, the $E_m$ is correlated with $R_{in}$ in both control ($\circ$) and ACh (●) conditions from the recordings of 19 cells in B1. Changes induced by ACh in $E_m$ and $R_{in}$ are shown in B2. None of the preceding relations is significantly correlated, suggesting that rather than passive membrane properties, the 5-HT and ACh induced changes in $E_m$ and $R_{in}$ may mainly result from modulation of active membrane properties of the EGFP+ neurons.
**Expression of c-fos-EGFP in spinal interneurons**

In addition to locomotor task, c-fos expression can be also induced by many other factors (Coggleshall 2005) such as cell death and surgical procedures in spinal neurons. To reduce these effects on our study, we have set several criteria to induce c-fos expression and to choose the EGFP+/H11001 neurons in this study: 1) the locomotor task (swimming) was induced in animals for 60–90 min. This long-duration, persistent, and nonstopping locomotor activity would allow spinal neurons driven by the locomotion to have enough time and intensity to express c-fos (Coggleshall 2005). In fact, our results confirmed the validity of this protocol for induction of the c-fos-EGFP expression in spinal interneurons. 2) Apoptosis is known to be associated with c-fos expression (Smeyne et al. 1993). In spinal interneurons, the death of cells due to apoptosis reached the peak at P0 and decreased rapidly by ~80% after P4 (Lowrie and Lawson 2000). For this reason, the animals we chose for patch-clamp experiments were all at (16%, 7/44) or older (84%, 37/44) than P4. This selection of animals could reduce the effect of apoptosis on c-fos-EGFP expression. 3) Although surgical procedures such as dissection and slicing process could induce c-fos expression, neurons expressing c-fos-EGFP by this way are usually unhealthy. This can be recognized by the shrinking cell body, visualized nuclear, rough surface, and ultra brightness of the EGFP expression. These cells were excluded for patch-clamp recording. In this study, only healthy neurons that were usually shown as medium (or sometimes weak) brightness of EGFP expression in target areas (laminar VII, VIII, and X) for patching the EGFP+/H11001 neurons. Although the number of EGFP+/H11001 neurons induced by nonlocomotor activity could be largely reduced by these criteria, we could not absolutely rule out the c-fos-EGFP expression triggered by some unwanted factors. The EGFP+/H11001 neurons that can be always seen in dorsal horn in both control and locomotor slices are such an example. As shown in Fig. 3, however, the pronounced increase in EGFP expression in ventral horn after swimming suggested that the locomotor activity indeed induced EGFP expression in spinal interneurons. These neurons are the target of the present study.

**Differences among the firing patterns, lamina distributions, and membrane properties**

A significant difference in R_in and C_m was shown to be correlated with neuronal laminar distribution but not type. This suggests differences in size and/or morphology of the activated neurons in the target areas. The active properties (V_th, AP, AHP, and F-I properties, etc), however, were not significantly related to the laminar distribution. Therefore the electrophysiological or functional differences in these neurons with respect to their role in locomotion is not clear.

**Overlapping of the EGFP+/H11001 neurons with some identified locomotor neurons**

The functional role of the EGFP+/H11001 neurons in locomotion is unknown. It is not possible to classify the EGFP+/H11001 neurons into any known type of locomotor interneuron given differences in the types of studies. However, the EGFP+/H11001 neurons and ascending commissural interneurons previously studied (Carlin et al. 2006; Zhong et al. 2006a,b) are modulated in a similar fashion by the neurotransmitters ACh and 5-HT. These similarities include the depolarization of membrane potential and reduction of input resistance by ACh, and the depolarization of membrane potential, reduction of AHP, hyperpolarization of V_th, reduction of rheobase, and increase of input resistance and action potential width by 5-HT. In addition, the target areas (laminar VII, VIII, and X) for patching the EGFP+/H11001 neurons are overlapped with the areas where the ascending commissural interneurons locate (Carlin et al. 2006; Eide et al. 1999). It is therefore possible that a proportion of the EGFP+/H11001 neurons studied here are ascending commissural interneurons.

**Membrane currents in EGFP+/H11001 neurons**

Two important ionic currents were found in EGFP+/H11001 neurons in the present study: hyperpolarization-activated inward currents and persistent inward currents. I_h has been shown to be involved in the production of bursting during various forms of rhythmic activity (Bertrand and Cazalets 1998; But et al. 2002; Kiehn et al. 2000; Thoby-Brisson et al. 2000) while PICs have been also shown to play an important role in rhythm generation in respiration (Del Negro et al. 2002, 2005) and locomotion (Tazerart et al. 2008; Zhong et al. 2007). The expression of I_h and PICs in EGFP+/H11001 neurons supports the importance of these two currents in the generation of locomotion. However, further studies are required to characterize I_h and PICs in EGFP+/H11001 neurons.

**5-HT-induced membrane oscillations**

5-HT-induced membrane oscillation was observed in EGFP+/H11001 neurons. Similar observations have been reported in recent studies in ascending commissural interneurons in neonatal mice (Carlin et al. 2006; Zhong et al. 2006a), where the 5-HT-induced membrane oscillations were shown to be either voltage dependent (Carlin et al. 2006) or independent (Zhong et al. 2006a) and could be blocked by TTX or the gap junction blocker carbenoxolone (Zhong et al. 2006a), suggesting that multiple mechanisms could be responsible for the oscillations. In this study, we demonstrated that in some cells, the frequency of the oscillations was voltage dependent (Fig. 7A) and could be persisted with APV and CNQX in the recording solution (B). These results indicate that intrinsic membrane properties play a role in generating the 5-HT-mediated membrane potential oscillations in some EGFP+/H11001 neurons, which may therefore have a role in rhythm generation. Membrane oscillation requires a coupling of inward and outward currents with precise occurrence of the currents in time. As suggested by many previous studies (e.g., Marder and Bucher 2001; Wilson et al. 2005), I_h, T-type calcium currents, and PICs (calcium and/or sodium components) are all candidates for induction of membrane oscillations in locomotor neurons. Enhancement of I_h and PICs by 5-HT have been reported in our recent studies (Dai and Jordan 2006; Dai et al. 2005a). Therefore the action of 5-HT on I_h and PICs could facilitate the membrane oscillation in EGFP+/H11001 neurons observed in this study. Further study is required to investigate this issue.

**Aminergic effects on EGFP+/H11001 neurons**

In this study, we demonstrate the modulation of membrane properties by 5-HT and ACh in EGFP+/H11001 neurons. Although
both agents increase the neuronal excitability, their effects on membrane properties are not exactly the same. Both agents induced paradoxical effects on membrane potential and hyperpolarized $V_{th}$. On the other side, however, 5-HT increased $R_{in}$ and reduced AHP, whereas ACh reduced $R_{in}$ and increased AHP. The variable effects of 5-HT and ACh on EGFP+ neurons could result in different mechanisms to regulate the neuronal excitability and output. 5-HT-induced changes in $F-I$ slope ($n = 7$) tended to be correlated to changes in $R_{in}$ (absolute value of correlation coefficient $R = 0.6$), AHP depth ($R = 0.6$), and $V_{th}$ ($R = 0.8$), whereas ACh-induced changes in $F-I$ slope ($n = 11$) were coupled relatively strongly to changes in $R_{in}$ ($R = 0.7$) and AP height and width ($R = 0.5$) but weakly to AHP duration ($R = 0.3$) and $V_{th}$ ($R = 0.2$). These differences in correlation reflected different levels of balance in modulatory forces driven by different mechanisms.

Conclusion

Using cfos-EGFP transgenic mice, we demonstrate the ability to study interneurons activated by a locomotor task. The capacity to select neurons active during locomotion adds a new method in which interneurons can be studied and may be particularly powerful when used in combination with other techniques such as retrograde labeling technique for study of commissural interneurons specifically activated by locomotion. Characterization of EGFP+ neurons located in lamina VII, VIII, and X is important to provide insight into the cellular basis for generation of locomotion. The membrane properties of these neurons and the manner in which they are modulated by 5-HT and ACh suggest that the EGFP+ neurons could play a role in generating or mediating locomotion.

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