5-HT Prolongs Ventral Root Bursting via Presynaptic Inhibition of Synaptic Activity During Fictive Locomotion in Lamprey

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Abstract

Locomotor pattern generation is maintained by integration of the intrinsic properties of the spinal central pattern generator (CPG) neurons in conjunction with synaptic activity of the neural network. In the lamprey, the spinal locomotor CPG is modulated by 5-HT. On a cellular level, 5-HT presynaptically inhibits synaptic transmission and postsynaptically inhibits a Ca$^{2+}$-activated K$^+$ current responsible for the slow afterhyperpolarization (sAHP) that follows action potentials in ventral horn neurons. To understand the contribution of these cellular mechanisms to the modulation of the spinal CPG, we have tested the effect of selective 5-HT analogues against fictive locomotion initiated by bath application of NMDA. We find that the 5-HT$_{1D}$ agonist, L694-247, dramatically prolongs the frequency of ventral root bursting. Furthermore, we demonstrate that L694-247 presynaptically inhibits synaptic transmission without altering postsynaptic Ca$^{2+}$-activated K$^+$ currents. We also confirm that 5-HT inhibits synaptic transmission at concentrations that modulate locomotion. To examine the mechanism by which selective presynaptic inhibition modulates the frequency of fictive locomotion we performed voltage and current clamp recordings of CPG neurons during locomotion. Our results show that 5-HT decreases glutamatergic synaptic drive within the locomotor CPG during fictive locomotion. Thus, we conclude that presynaptic inhibition of neurotransmitter release contributes to 5-HT-mediated modulation of locomotor activity.

Key Words: Central Pattern Generator (CPG), NMDA oscillations, glutamate release, Serotonin, Spinal Cord
Introduction

Central pattern generators (CPGs) drive rhythmic motor activities such as locomotion (Grillner 2003; Grillner and Wallen 2002), respiration (Del Negro et al. 2002), and feeding (Marder 1994). The neuronal correlates to these behaviors are produced by integrating the intrinsic properties of the CPG neurons in conjunction with synaptic activity of the neural network (Alford et al. 2003; Marder and Thirumalai 2002). Several endogenous neurotransmitters have been shown to alter the output of locomotor CPGs and to modulate cellular and synaptic properties of CPG neurons (Alford et al. 2003; Barbeau and Rossignol 1991; Grillner and Wallen 2002; McLean et al. 2000; Parker 2000; Perrier et al. 2003; Schotland et al. 1996; Svensson et al. 2003). However, less is known about the mechanisms by which these cellular and synaptic changes modulate the output of the CPG. We have investigated the contribution of 5-HT-mediated presynaptic inhibition of synaptic transmission to modulating the lamprey locomotor network.

The neural network of the lamprey locomotor CPG has been well characterized. It is maintained by ipsilateral glutamatergic excitation in conjunction with contralateral inhibition, (Alford and Williams 1989; Brodin et al. 1985; Buchanan 1982; Buchanan and Grillner 1991; Grillner and Wallen 1980; Hellgren et al. 1992) and comprises ventral root bursting that alternates across the spinal cord (Grillner et al. 1995). Lamprey spinal CPGs are activated by glutamate release from brain stem reticulospinal (RS) neurons (Buchanan et al. 1987; Buchanan and Cohen 1982; Ohta and Grillner 1989). The intensity of input from reticulospinal axons regulates the frequency of these bursts of activity and therefore the speed of locomotion (Brocard and Dubuc 2003; Di Prisco et al. 2000), which may range from 0.1 to 10 Hz. Experimentally, locomotor CPG activity in the spinal cord may also be activated by electrical stimulation of the lamprey brainstem in semi-intact preparations (McClellan and Grillner 1984; Sirota et al. 2000) or by
application of glutamate receptor agonists in isolated spinal cords (Cohen and Wallen 1980; Grillner et al. 1981). The alternating pattern of ventral root bursting recorded under these experimental conditions is referred to as fictive locomotion (Grillner 2003), and is thought to drive the coordinated contraction of muscles necessary for lamprey swimming.

The frequency of fictive locomotion is modulated by endogenous release of neurotransmitters within the spinal cord (Christenson et al. 1989; Harris-Warrick and Cohen 1985; Parker 2000; Parker and Grillner 1998, 1999; Schotland et al. 1996; Svensson et al. 2003). Of these modulatory neurotransmitters, 5-HT reduces the frequency of ventral root bursting during fictive locomotion (Harris-Warrick and Cohen 1985). This is believed to be due, in part, to 5-HT-mediated inhibition of a postsynaptic Ca^{2+}-dependant K^{+}-current (I_{K(Ca)}) that underlies the slow after-hyperpolarization (sAHP) of action potentials in neurons of the CPG (El Manira et al. 1994; Parker and Grillner 2000; Wallen et al. 1989; Wikstrom et al. 1995). Inhibition of a postsynaptic I_{K(Ca)} is thought to play a role in prolonging fictive locomotion bursts through 1) increasing the number of spikes per burst by shortening the sAHP and 2) prolonging the plateau of NMDA TTX oscillations (Christenson et al. 1989; El Manira et al. 1994; Schotland and Grillner 1993; Wallen et al. 1989; Wallen and Grillner 1987). Computer models suggest that inhibition of I_{K(Ca)} in neurons within the lamprey CPG prolongs fictive locomotion ventral root bursting (Hellgren et al. 1992; Lansner and Ekeberg 1994; Tegner et al. 1998). Apamin, a selective I_{K(Ca)} channel antagonist, has been used to test this hypothesis but with conflicting results. The finding that apamin significantly prolongs ventral root bursting during fictive locomotion (Hill et al. 1992) has been directly contradicted (Meer and Buchanan 1992). These differing results were clarified to some degree when it was suggested that the effect of apamin on fictive locomotion was dependent on the frequency of ventral root bursting. At lower burst frequencies apamin
significantly prolongs ventral root bursting, whereas at higher bursting frequencies the
effect of apamin is not significant (Buchanan 2001; Grillner et al. 2001).

5-HT alters the output of CPGs in several species (Schmidt and Jordan 2000). In
adult chronic spinal (t13) cats, 5-HT modulates treadmill induced locomotor patterns
(Barbeau and Rossignol 1990; Edgerton et al. 1997) and depresses the sAHP in
motorneurons (White and Fung 1989). Application of 5-HT modulates fictive locomotion
in turtles, presumably by activation of plateau potentials by 5-HT2 receptors (Alaburda et
al. 2002) and inhibition of K+ conductance by 5-HT1A receptors (Perrier et al. 2003).

In addition to activating a postsynaptic I_{K(Ca)}, 5-HT presynaptically inhibits
synaptic transmission in the lamprey spinal cord (Blackmer et al. 2001; Buchanan and
Grillner 1991; El Manira et al. 1994; Shupliakov et al. 1995; Takahashi et al. 2001). As in
lamprey, inhibition of synaptic transmission by 5-HT has been observed in several
vertebrate CPGs. 5-HT presynaptically inhibits midcycle glycineric inputs and prolongs
ventral root bursting during Xenopus larval swimming (Sillar et al. 1998). In neonatal rat,
activation of 5-HT receptors presynaptically decreases inspiratory modulated synaptic
currents (Di Pasquale et al. 1997; Hilaire et al. 1997; Lindsay and Feldman 1993) and
suppresses descending glutamatergic responses (Skagerberg and Bjorklund 1985).
Furthermore, both glutamatergic and glycineric synaptic transmission to rat hypoglossal
motorneurons is inhibited by activation of 5-HT1B receptors (Singer et al. 1996; Umemiya
and Berger 1995). In spite of this evidence, less in known about the contribution of
presynaptic inhibition of synaptic transmission to the modulation of the CPG output. We
now demonstrate in the lamprey that the 5-HT1D agonist, L694-247, acts presynaptically
to inhibit synaptic transmission without activating the known postsynaptic locus. Using
this selective 5-HT1D agonist, we show that presynaptic inhibition of synaptic
transmission is sufficient to slow the rhythm of fictive locomotion. Furthermore, our
results reveal that 5-HT modulates locomotor activity during fictive locomotion by presynaptically decreasing glutamatergic synaptic drive within the CPG.
Methods

The lamprey preparation

Experiments were performed on isolated spinal cords of both adult and larval lampreys (Petromyzon marinus and Ichthyomyzon unicuspis). The animals were anesthetized with tricaine methanesulfonate (MS222), decapitated in accordance with institutional guidelines, and dissected in a cold saline solution (Ringer) of the following composition (in mM): 100 NaCl, 2.1 KCl, 2.6 CaCl₂, 1.8 MgCl₂, 4 glucose, 26 NaHCO₃; bubbled with 95% O₂/5% CO₂ to a pH of 7.60 (modified from Wickelgren 1977). The spinal cord (12 to 20 segments) was isolated and removed from the protective meninx primitiva and placed in a cooled small-volume Sylgard-lined chamber. The recording chamber was continually superfused with cold oxygenated Ringer (8°C-10°C) or solutions of pharmacological agents bath-applied at a perfusion rate of approximately 1ml/min. In experiments involving whole cell patch recording, a 10 to 20 μm slice of tissue was removed from the surface of the spinal cord superior to the ventral horn using a vibrotome tissue slicer. Patch pipettes were then readily introduced to the cut ventral surface. Fictive locomotion was induced by application of 100 to 150 μM NMDA.

Electrophysiology

Ventral horn neurons (motoneurons or interneurons) were whole-cell clamped (with an Axopatch 200A amplifier, Axon Instruments) using a modified blind technique (Blanton et al. 1989; Cochilla and Alford 1997). Cell types were identified by their location in the tissue and neurons were distinguished from the non-neuronal cells and axons by their membrane properties and their capacitive transients in response to a 10 ms 10 mV step. Paired recordings were made between presynaptic reticulospinal axons and
postsynaptic spinal neurons, and action potentials were evoked in the presynaptic axons at 15s intervals. Pipettes had open-tip resistances of 5-10 MΩ. Series resistance was monitored continuously by giving a 10 mV voltage step before each episode, and if the change exceeded 15%, the cell was discarded. Microelectrode (sharp) recordings were made conventionally with thin walled glass. Tip resistances of 20 to 50 MΩ when filled with 3M potassium methane sulfonate allowed recording from either post-synaptic somata or presynaptic axons. Ventral root recordings were performed with glass extracellular suction electrodes and amplified with a differential AC amplifier from A-M systems.

Solutions

Patch pipette solution contained (mM): caesium methane sulphonate 102.5, NaCl 1, MgCl$_2$ 1, EGTA 5, HEPES 5, pH adjusted to 7.2 with CsOH. Microelectrode pipette solution was either 3 M potassium methane sulphonate or 3M potassium acetate. External solution contained (mM) NaCl 100, KCl 2.1, CaCl$_2$ 2.6, MgCl$_2$ 1.8, NaHCO$_3$ 26, glucose 4; bubbled with 95% O$_2$/5% CO$_2$. NMDA and all 5-HT analogues were obtained from Tocris; all other chemicals were from Sigma unless otherwise noted. Drugs were applied to the superfusate or applied over the spinal cord by pressure ejection from a fine pipette (patch pipette) with a 200 to 800 ms pulse of pressure (100 kPa).

Data analysis

For paired recordings, the mean was taken of at least 12 traces of EPSCs for each condition for each animal. For biphasic ESPCs, the decay of the electrical component
was estimated by fitting an exponential to the visible portion of the electrical component. The fit was then subtracted from the entire EPSC leaving the chemical component to determine the peak amplitude.

**Statistics**

Data are given as means ± S. E. M. *Student* paired two-tailed t-test was used to calculate the significance of the data, unless otherwise noted.
Results

Effect of L694-247 on fictive locomotion

Application of 5-HT to the lamprey spinal cord prolongs ventral root bursting and is known to activate at least two pharmacologically distinct receptors (El Manira et al. 1997; Wikstrom et al. 1995). One receptor, identified as 5-HT$_{1A}$-like, inhibits a $I_{K(Ca)}$ (Wikstrom et al. 1995), but does not alter synaptic transmission at sensory synapses in the lamprey spinal cord (El Manira et al. 1997). A second unclassified 5-HT receptor is known to presynaptically inhibit synaptic transmission at several sites in the spinal cord, including the reticulospinal synapse and sensory inputs (Blackmer et al. 2001; Buchanan and Grillner 1991; El Manira et al. 1994; Shupliakov et al. 1995; Takahashi et al. 2001). Thus, to study the effect of 5-HT mediated presynaptic inhibition of synaptic transmission on fictive locomotion, it is necessary to identify a specific agonist that selectively inhibits synaptic transmission without activating postsynaptic 5-HT receptors that inhibit $I_{K(Ca)}$.

Previous studies have shown that the action of 5-HT to inhibit postsynaptic $I_{K(Ca)}$ prolongs ventral root bursting during fictive locomotion(El Manira et al. 1994; Grillner et al. 2001; Wallen et al. 1989). Should presynaptic inhibition of synaptic transmission also contribute to modulation of fictive locomotion, we would expect that selectively activating presynaptic receptors that inhibit synaptic transmission would also prolong ventral root bursting. To investigate this hypothesis, we assayed the effects of various 5-HT receptor agonists and antagonists versus fictive locomotion. Simultaneous recordings of contralateral ventral roots were made to monitor fictive locomotion induced by 100 to 150 $\mu$M NMDA (Figure 1 Ai, Bi). Of all the 5-HT analogues tested only the 5HT$_{1D}$ agonist, L694-247, significantly prolonged fictive locomotion ventral root bursting (Table 1). In 11 of 17 preparations the burst duration, interburst interval, and frequency of
bursts, were significantly prolonged (Figure 1 Ai, Aii). Of the 6 experiments in which ventral root bursting was not prolonged, L694-247 caused the ventral root output to become disorganized in 3 experiments, preventing analysis of the bursts (Figure 1 Bi, Bii). In the remaining 3 experiments, L694-247 did not significantly alter ventral root bursting. An average of all analyzed experiments demonstrated that 100 nM L694-247 significantly prolonged burst duration (216 ± 38 % of control), interburst interval (215 ± 29 % of control), and reduced the frequency of bursts (59 ± 8 % of control, S.E.M., Figure 1, paired student t-test, p-values < 0.05, for all variables). Thus, the 5-HT$_{1D}$ agonist L694-247 causes a marked prolongation of burst duration and reduction in burst frequency, similar to the effect of 5-HT (Harris-Warrick and Cohen 1985) and of the 5-HT$_{1A}$ agonist 8-OH-DPAT ((+/-)-8-hydroxy-dipropylaminotetralin hydrobromide) (Wikstrom et al. 1995).

Despite the profound effect of the 5-HT$_{1D}$ agonist L694-247, the 5-HT$_{1D}$ antagonist, BRL 15572, did not alter either fictive locomotion in control conditions (100 nM, 500 nM, n=4) or prevent 5-HT$_{1D}$ agonist-mediated prolonged bursting (100 nM, 1µM n=5). We were also unable to antagonize the effect of L694-247 on locomotion with 5-HT$_{2}$ antagonist Cyproheptadine (20, 100 and 500 µM; n=6) or 5-HT$_{1B}$ antagonist SB216641 (5 nM; n=3) (Table 1). Although these results are inconclusive, they are consistent with results of 5-HT in rabbit. Inhibition of the sural-gastronemius monosynaptic reflex pathway in the rabbit by exogenous 5-HT application is thought to be mediated by 5-HT$_{1D_{,x}}$ or 5-HT$_{2}$ receptors, but a specific 5-HT antagonist has yet to be identified and has led some to propose the effect is mediated by a novel receptor (Schmidt and Jordan 2000).
5-HT\textsubscript{1D} agonist, L694-247, inhibits neurotransmitter release at the RS synapse

To probe the site of action of this 5-HT\textsubscript{1D} agonist, we used the RS synapse to assay the effects of L694-247 on synaptic transmission. The lamprey spinal preparation is uniquely advantageous for studying synaptic transmission electrophysiologically since descending RS axons are un-myelinated and form en passant synapses. The lack of myelin allows for microelectrode penetration of presynaptic axons. Moreover, recording from anywhere along the length of an en passant axon is electrophysiologically equivalent to recording directly from the terminal. Thus, the lamprey RS synapse is one of the few known synapses that allow electrophysiological access to both the pre- and postsynaptic neurons.

To assay the effect of L694-247 on synaptic transmission, paired recordings were made between presynaptic RS axons and postsynaptic spinal neurons (Figure 2A). Action potentials evoked in the presynaptic RS axon (Figure 2B) resulted in biphasic EPSCs, containing both electrical and chemical components (Figure 2C). The initial fast invariant phase of the EPSC is consistent with a current carried through gap junctions (this electrical component was observed in most, but not all recordings), while the slower variable phase is mediated through synaptic release of glutamate and activation of AMPA and NMDA receptors (Figure 2C) (Buchanan et al. 1987). We found that the 5-HT\textsubscript{1D} agonist, L694-247 (100 nM), inhibited the chemical component of evoked EPSCs to 37 ± 16\% of control (Figure 2C, S.E.M., p< 0.05, n=3). L694-247 did not alter either the impedance of the cell or the electrical component of the EPSCs, consistent with a presynaptic mechanism of inhibition (Figure 2C). This result is in accord with the conclusions of previous studies that 5-HT presynaptically inhibits synaptic transmission (Blackmer et al. 2001; Buchanan and Grillner 1991; El Manira et al. 1994; Shupliakov et al. 1995; Takahashi et al. 2001). Thus, L694-247, a specific 5-HT\textsubscript{1D} agonist, inhibits
chemical synaptic transmission at this synapse in a manner similar to the known presynaptic effect of 5-HT.

Since we were unable to antagonize the effect on L694-247 on locomotion, we probed for a specific antagonist to 5-HT-receptor mediated inhibition of synaptic transmission. Nevertheless, neither SB224289 (5-HT$_{1B}$ antagonist) nor GR 55562 (5-HT$_{1D\beta}$ antagonist) reversed inhibition of synaptic transmission mediated by 1 µM 5-HT (Table 1).

**5-HT$_{1D}$ agonist, L694-247, does not inhibit the postsynaptic sAHP**

In addition to presynaptically inhibiting synaptic transmission, 5-HT also inhibits a postsynaptic apamin sensitive Ca$^{2+}$ dependent K$^+$-current ($I_{K(Ca)}$) (El Manira et al. 1994; Wallen et al. 1989; Wikstrom et al. 1995). To test whether this 5-HT$_{1D}$ agonist (L694-247) inhibits postsynaptic $I_{K(Ca)}$, microelectrode recordings were made from spinal neurons. We rapidly applied L694-247 by pressure ejection since subtle changes in the membrane potential that occur during prolonged bath application of drugs are capable of altering the sAHP. Two pressure ejection pipettes, filled with either 5-HT (300 µM) or L694-247 (1 µM), were positioned within 1 mm of the recording pipette. To compensate for dilution of L694-247 during pressure ejection we used a concentration 100 fold higher than the Kd (Glennon et al. 1996) (10 fold higher than a dose that significantly inhibits synaptic transmission, see Figure 2). To visualize ejection of the drugs, the dye Fast Green (Fisher) was included in the pressure ejection pipettes and the spread of dye from the pipette was observed through a stereo-microscope. Fifteen action potentials were evoked at 0.5 Hz by brief current injection. Following the 4$^{th}$ action potential either 5-HT or L694-247 was alternately pressure ejected (200 to 800 ms, 100 kPa). Comparison of the sAHP in control versus in L694-247, demonstrated that L694-247 did not alter the sAHP (Figure 3 Bi, Bii, 103% ± 0.5 of control, S.E.M. p>0.05, n=3). This result is
consistent with the findings of other studies, which demonstrated that the 5-HT\(_{1D}\) agonist, sumatriptan, also did not alter the sAHP (Wikstrom et al. 1995). As previously demonstrated, 5-HT inhibited the sAHP (Figure 3 Ai, Aii, 34% ± 12 of control, S.E.M. p<0.05, n=3)(Wallen et al. 1989). Thus, the 5-HT\(_{1D}\) agonist, L694-247 acts at the presynaptic locus of 5-HT without affecting the known postsynaptic locus.

**5-HT inhibits synaptic transmission in a dose-dependent manner**

To understand the activity of endogenous 5-HT on fictive locomotion, we assayed the apparent affinity of 5-HT at the presynaptic locus by making paired recordings at the RS synapse (as described above) in the presence of varying doses of 5-HT. Action potentials evoked in the presynaptic RS axon resulted in EPSCs recorded in motor neurons (Figure 4A). We found that 5-HT inhibited synaptic transmission in a dose dependent manner (Figure 4B, 600nM = 48.65% ± 8.05 n=9, 100 nM = 63.11% ± 17.46 of control. n=3. S.E.M. p< 0.05). Furthermore, saturating doses of 5-HT inhibited synaptic transmission to approximately 20% of control (Figure 4B, 1 \(\mu\)M = 16.9 ± 6.6 n=5, 30 \(\mu\)M = 19.8 ± 7.5 of control. n=4. S.E.M. p<0.05). By generating a dose response curve and fitting the data with a Hill plot, we found that 5-HT inhibits synaptic transmission with an apparent affinity of 143.5 ± 75 nM (Figure 4B). This is a significantly higher apparent affinity compared to previous reports which found that 5-HT presynaptically inhibits synaptic transmission with an apparent \(K_d\) of 2.3 \(\mu\)M and a maximal inhibition of 49% of control (30 \(\mu\)M 5-HT) (Takahashi et al. 2001). The dichotomy is likely due to previous studies using extracellular stimulation to evoke action potentials in a mixed population of both excitatory and inhibitory axons in the spinal cord. Additionally, the different location of axons within the spinal cord may affect the concentration of 5-HT that reaches the synapse. For example, RS synapses are very
close to the spinal cord surface, whereas axons embedded deeper in the tissue are likely to be protected by endogenous 5-HT uptake. Our findings demonstrate a similar apparent affinity of 5-HT for the presynaptic locus as for modulation of fictive locomotion (~100 nM) (Harris-Warrick and Cohen 1985).

**How might inhibition of neurotransmitter release alter fictive locomotion?**

5-HT inhibits synaptic transmission at the lamprey RS synapse. Specific activation of presynaptic 5-HT$_{1D}$-like receptors inhibits synaptic transmission and leads to a prolongation of burst duration during fictive locomotion. This result is consistent with the effects of 5-HT itself on fictive locomotion. However, probing the effects of L694-247 on synaptic transmission at the RS synapse has indirect implications on the effect of 5-HT during locomotion since this synapse is quiescent during fictive locomotion stimulated by bath applied NMDA. It is therefore important to study the effect of 5-HT (1 μM) on monosynaptic projections from intraspinal excitatory interneurons (EIN) to other neurons of the central pattern generator. Unfortunately, stable recordings of EINs are hard to maintain and positive identification of these cells is difficult. Alternatively, it is possible to identify and directly evaluate excitatory synaptic drive from EINs by voltage clamping spinal ventral horn neurons during fictive locomotion.

During fictive locomotion driven by bath application of NMDA, membrane potential oscillations of any recorded neuron are driven both by synaptic drive from excitatory and inhibitory interneurons and from activation of NMDA receptors directly from the bath applied NMDA. We hypothesize that 5-HT significantly inhibits excitatory drive during fictive locomotion, but that it will not alter NMDA-receptor mediated membrane potential oscillations, which will continue to be activated by the bath applied NMDA. Indeed, in cells recorded under current clamp conditions, the addition of 5-HT to the superfusate significantly reduced the frequency of fictive locomotion but did not alter
the amplitude of membrane potential oscillations (Figure 5Ai, Aii, n=10). To characterize the currents that underlie these slower membrane potential oscillations, we voltage clamped the ipsilateral neurons at -65 mV and recorded current oscillations. Voltage clamping the cell inhibits NMDA receptor-mediated current oscillations, as membrane potential excursions needed for Mg\(^{2+}\) un-block of the receptor channel are no longer possible. Furthermore, we do not expect Cl\(^-\) currents contribute to current oscillations since –65 mV is near the Cl\(^-\) reversal potential (Grillner and Wallen 1980). Thus, any remaining depolarizing current oscillations observed under voltage clamp would be predominantly mediated by AMPA receptors. Under voltage clamp conditions, current oscillations observed during fictive locomotion were abolished by addition of 1 µM 5-HT (n=3), indicating that 5-HT inhibits AMPA mediated synaptic currents during fictive locomotion (Figure 5Bi, 5Bii). Furthermore, the slower membrane potential oscillations that persist under current clamp in 5-HT are likely driven by NMDA receptor currents activated by bath applied NMDA.
Discussion

Locomotor CPGs integrate the intrinsic oscillatory properties of spinal neurons with the synaptic activity of the network to produce coordinated neuromuscular excitation and movement. The frequency of fictive locomotion is slowed by application of 5-HT in the lamprey (Harris-Warrick and Cohen 1985). On a cellular level, 5-HT is known to activate two distinct receptors. Postsynaptically, activation of 5-HT$_{1A}$ receptors causes inhibition of an apamin sensitive $I_{K(Ca)}$. Inhibiting this current is thought to prolong ventral root bursting during fictive locomotion by blocking the sAHP of spinal neuron action potentials (El Manira et al. 1994; Wallen et al. 1989; Wikstrom et al. 1995). Additionally, activation of presynaptic 5-HT receptors inhibits synaptic transmission, but the contribution of this 5-HT mediated inhibition on prolonging ventral root bursting during locomotion has not previously been investigated.

While it is known that activation of 5-HT receptors markedly slows fictive locomotion, the site of action of 5-HT may be postsynaptic, presynaptic or both. We now demonstrate that a selective 5-HT$_{1D}$ receptor agonist (L694-247) slows fictive locomotion, qualitatively mimicking the effects of 5-HT and of a selective 5-HT$_{1A}$ agonist known to inhibit postsynaptic $I_{K(Ca)}$.

At the same dose (100 nM) that profoundly reduced the frequency of fictive locomotion, L694-247 markedly inhibits synaptic transmission, but leaves unaffected the sAHP of postsynaptic action potentials. There is extensive evidence that 5-HT acts presynaptically to inhibit synaptic transmission in the lamprey spinal cord. 5-HT mediated inhibition of synaptic responses is prevented by selectively blocking G-protein signaling in presynaptic terminals (Blackmer et al. 2001). Furthermore, it is unlikely that 5-HT acts postsynaptically to alter synaptic currents since postsynaptic responses to pressure ejection of glutamate are not affected by doses of 5-HT that inhibit synaptic transmission (Buchanan and Grillner 1991). Additionally, both the impedance of
postsynaptic neurons and the electrical component of EPSCs are left unaffected by 5-HT (El Manira et al. 1997; Takahashi et al. 2001). Since L694-247 is a 5-HT receptor agonist and does not alter either the electrical component of the EPSC or the input impedance of the postsynaptic cell, it follows that L694-247 inhibits synaptic transmission by activating the previously identified presynaptic 5-HT receptor.

Interestingly, L694-247 did not exhibit the same effect on fictive locomotion in all experiments. Application of L694-247 led to a disorganized pattern of ventral root bursting in 3 of the preparations. If selective activation of presynaptic 5-HT receptors inhibits glutamate release, then we may consider a number of alternative possible outcomes to its effect on fictive locomotion. First, we must consider the method of activation used to maintain fictive locomotion. In the isolated lamprey spinal cord this has generally been achieved by bath application of a glutamate receptor agonist, typically either NMDA or D-glutamate. Either of these methods will effectively activate postsynaptic NMDA receptors. NMDA stimulates fictive locomotion by a direct action as an agonist, whereas D-glutamate acts by inhibiting glutamate uptake and by raising intraspinal extracellular glutamate concentrations to levels that will primarily activate NMDA receptors because of their relatively high affinity to glutamate (Dunlop 2001). In addition, D-glutamate also acts as a weak NMDA receptor agonist (Olverman et al. 1988). Thus, fictive locomotion activated in this way will be driven by a combination of NMDA receptor-mediated membrane potential oscillations which are augmented and phase-locked by excitatory and inhibitory synaptic drive (Grillner 2003). If synaptic drive were severely inhibited by 5-HT, then we may hypothesize that spinal neurons, while still excited by NMDA, would no longer be phase-locked by synaptic drive and their ventral root output would be random. We believe this was the paradigm we observed in the three preparations where L694-247 caused ventral root bursting to become disorganized. However, the mean reduction in synaptic transmission mediated by L694-
247 was approximately 37% of control. We may hypothesize that under these more moderate inhibitory conditions the frequency of fictive locomotion will be coupled with the remaining synaptic drive, but will be dominated by the resonant frequency of the NMDA receptor-mediated membrane potential oscillations driven by bath application of NMDA. In fact, such an outcome has been directly predicted by computer models of fictive locomotion in the lamprey spinal cord (Hellgren et al. 1992; Lansner and Ekeberg 1994) and is supported by physiological studies of the effects of glutamatergic agonists and antagonists on fictive locomotion (Brodin and Grillner 1986; Brodin et al. 1985). Indeed, we show clearly that selective activation of a presynaptic 5-HT1D receptor very significantly slows fictive locomotion. We, therefore, conclude that 5-HT slows fictive locomotion by reducing synaptic release of glutamate in the spinal cord.

This conclusion is predicated on a model in which 5-HT activates presynaptic receptors to inhibit transmitter release from CPG neurons during fictive locomotion. Thus 5-HT must inhibit synaptic transmission at doses of 5-HT that also prolong ventral root bursting. By generating a dose response curve of 5-HT versus inhibition of synaptic transmission we demonstrate that 5-HT inhibits synaptic release of glutamate with an apparent Kd of 140 nM. This dose is entirely consistent with doses of 5-HT known to modulate fictive locomotion (Harris-Warrick and Cohen 1985). We have also demonstrated that 5-HT significantly reduces excitatory synaptic drive onto neurons of the CPG during NMDA-activated fictive locomotion. Thus, neurons show rhythmic membrane potential oscillations during fictive locomotion in NMDA in phase with ipsilateral ventral root bursting activity and similarly demonstrate these oscillations in the presence of 5-HT. However, when we whole cell voltage clamp the neurons to isolate AMPA-mediated rhythmic synaptic drive, addition of 5-HT blocks this synaptic drive and we see no further rhythmic oscillations in current across the cell membrane.
**Acknowledgements:** We express our gratitude to Rejean Dubuc, Trillium Blackmer, Huzefa Photowala, Sid Ramakrishnan and Caroline Marty for their very helpful discussions and comments on the manuscript. This work was supported by NINDS NS31713 and NSF IBN 0094444 grants.


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FIGURE 1. Activation of 5-HT₁D-like receptors slows the frequency of NMDA-induced fictive locomotion. Application of NMDA (100-150 µM) induced fictive locomotion (Ai, Bi). Fictive locomotion ventral root bursting was monitored by performing extracellular ventral root recordings on opposite sides of the spinal cord (vr1, vr2). In 11 of 17 animals, application of the 5-HT₁D agonist, L694-247 (100 nM), slowed the frequency of fictive locomotion and prolonged ventral root bursting (Aii). In 3 of the 17 animals L694-247 caused the ventral root bursting pattern to become disorganized (Bii).

FIGURE 2. 5-HT₁D agonist, L694-247, inhibits synaptic transmission at the RS synapse. A) Schematic diagram of the recording setup for synaptically paired neurons. The presynaptic reticulospinal axon is recorded from with a microelectrode and the postsynaptic spinal neuron is recorded from with a patch electrode. Action potentials evoked by injecting current through the presynaptic microelectrode (B) evoked biphasic EPSCs in spinal neurons (C). Typical trials are displayed before (black) and after (grey) the application of L694-247. 100 nM L694-247 inhibited the chemical component of synaptic transmission to 37 ± 16 % of control (grey, C). These data represent the mean of 12 consecutive trials. Application of L694-247 did not alter the input impedance or the electrical component of the EPSC (C).

FIGURE 3. 5-HT₁D agonist, L694-247, does not activate postsynaptic 5-HT receptors that mediate inhibition of the AHP of action potentials. Microelectrode recordings were made in spinal neurons. Fifteen action potentials were elicited every 3 s by a brief (2 ms) intracellular current pulse. Pressure ejection (15 psi, 200 to 800 ms) of either 5-HT (100 µM, Ai) or L694-247 (1 µM, Bi) was giving adjacent to the recording electrode following the 4th action potential (arrow). Comparison of the AHP in control, the 4 action potentials prior to the puff, and in L694-247, the last 4 actions potentials, demonstrated
that L694-247 did not alter the AHP (Bi, 103% ± 0.05, n=3). As previously shown, 5-HT inhibited the AHP (Ai, 34% ± 12, n=3). Aii, Bii, expanded average of 4 sAHP before (black) and after pressure ejection of either 5-HT or L694-247 respectively (grey).

**FIGURE 4.** Characterization of 5-HT mediated inhibition of synaptic transmission at the RS synapse. A) A presynaptic action potential was evoked in the RS axon by current injection (2 ms) through the microelectrode. This evoked EPSCs recorded through the patch clamp electrode. 5-HT (1 µM) reduces the chemical component of the EPSC (grey). B) Dose response curve of synaptic transmission versus 30 µM, 1 µM, 600 nM, and 100 nM 5-HT. Fitting the data with a Hill plot demonstrated 5-HT inhibits synaptic transmission with an apparent Kd of 143 ± 75 nM, with saturating doses causing a maximal inhibition to ~ 20 % of control.

**Figure 5.** 5-HT inhibits synaptic drive from excitatory interneurons (EINs) to ventral horn spinal neurons during fictive locomotion. Microelectrode recordings of spinal locomotor CPG neurons and extracellular ventral root recordings were made during fictive locomotion maintained by bath application of NMDA (100 to 150 µM). Under current clamp conditions (Ai) the membrane potential oscillated in phase with ipsilateral ventral root bursting. Following application of 1 µM 5-HT both the membrane potential oscillations and the ventral root bursts were prolonged (Aii). To isolate synaptic drive mediated by AMPA receptors, spinal neurons were voltage clamped at –65 mV (Bi). This is near the reversal potential for glycinergic Cl⁻ conductance, and we therefore do not expect Cl⁻ to play a large role in shaping these current oscillations. Under these conditions, depolarizing current oscillations observed during fictive locomotion are
predominantly mediated by AMPA receptors. Application of 5-HT (1 µM) abolished these current oscillations (Bii).
Table 1. Effect of 5-HT analogues on fictive locomotion

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Name of Drug</th>
<th>Concentration</th>
<th>n</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₁D Agonist</td>
<td>L694-247</td>
<td>100 nM</td>
<td>n=17</td>
<td>Prolonged Bursting*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 nM</td>
<td>n=7</td>
<td>No Effect on Locomotion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 nM</td>
<td>n=1</td>
<td>No Effect on Locomotion</td>
</tr>
<tr>
<td></td>
<td>BRL 15572</td>
<td>1 µM</td>
<td>n=1</td>
<td>No Effect on Locomotion</td>
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<tr>
<td>5-HT₁D Antagonist</td>
<td>Cyproheptadine</td>
<td>20 µM</td>
<td>n=6</td>
<td>No Effect on Locomotion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 µM</td>
<td>n=6</td>
<td>No Effect on Locomotion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 µM</td>
<td>n=2</td>
<td>No Effect on Locomotion</td>
</tr>
<tr>
<td>5-HT₂ Antagonist</td>
<td>SB216641</td>
<td>5 nM</td>
<td>n=3</td>
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<tr>
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<td>1 µM</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>10 µM</td>
<td>n=1</td>
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<tr>
<td>5-HT₁B Antagonist</td>
<td>SB224289</td>
<td>60 nM</td>
<td>n=2</td>
<td>No Effect on Syn.Trans.**</td>
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<tr>
<td></td>
<td></td>
<td>300 nM</td>
<td>n=3</td>
<td>No Effect on Syn.Trans.**</td>
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<tr>
<td>5-HT₁/₇B Antagonist</td>
<td>GR 55562</td>
<td>500 nM</td>
<td>n=3</td>
<td>No Effect on Syn.Trans.**</td>
</tr>
</tbody>
</table>

* Represents p-value < 0.05; No effect = p-value > 0.05. **Drugs did not to reverse inhibition of synaptic transmission by 1 µM 5-HT.
FIGURE 1
FIGURE 3
FIGURE 4
Figure 5