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Serotonin 5-HT$_2$ Receptors Induce a Long-Lasting Facilitation of Spinal Reflexes Independent of Ionotropic Receptor Activity

Barbara L. Shay, Michael Sawchuk, David W. Machacek, and Shawn Hochman

1Department of Physical Therapy, University of Manitoba, Winnipeg, Manitoba, Canada; and 2Department of Physiology, Emory University, Atlanta, Georgia

Submitted 6 May 2005; accepted in final form 14 July 2005

Shay, Barbara L. Michael Sawchuk, David W. Machacek, and Shawn Hochman. Serotonin 5-HT$_2$ receptors induce a long-lasting facilitation of spinal reflexes independent of ionotropic receptor activity. J Neurophysiol 94: 2867–2877, 2005. First published July 20, 2005; doi:10.1152/jn.00465.2005. Dorsal root-evoked stimulation of sensory afferents in the hemisected in vitro rat spinal cord produces reflex output, recorded on the ventral roots. Transient spinal 5-HT$_{2C}$ receptor activation induces a long-lasting facilitation of these reflexes (LLFR) by largely unknown mechanisms. Two Sprague–Dawley substrains were used to characterize network properties involved in this serotonin (5-HT) receptor–mediated reflex plasticity. Serotonin more easily produced LLFR in one substrain and a long-lasting depression of reflexes (LLDR) in the other. Interestingly, LLFR and LLDR were bidirectionally interconvertible using 5-HT$_{2A/2C}$ and 5-HT$_{1A}$ receptor agonists, respectively, regardless of substrain. LLFR was predominantly A$eta$ afferent fiber mediated, consistent with prominent 5-HT$_{2C}$ receptor expression in the A$eta$ fiber projection territories (deeper spinal laminae). Reflex facilitation involved an unmasking of polysynaptic pathways and an increased receptive field size. LLFR emerged even when reflexes were evoked three to five times/h, indicating an activity independent induction. Both the NMDA and AMPA/kainate receptor–mediated components of the reflex could be facilitated, and facilitation was dependent on 5-HT receptor activation alone, not on coincident reflex activation in the presence of 5-HT. Selective blockade of GABA$_A$ and/or glycine receptors also did not prevent reflex amplification and so are not required for LLFR. Indeed, a more robust response was seen after blockade of spinal inhibition, indicating that inhibitory processes serve to limit reflex amplification. Overall we demonstrate that the serotonergic system has the capacity to induce long-lasting bidirectional changes in reflex strength in a manner that is nonassociative and independent of evoked activity or activation of ionotropic excitatory and inhibitory receptors.

INTRODUCTION

Bulbospinal serotonergic systems potently modulate spinal cord sensory and motor activity (Hochman et al. 2001; Millan 2002; Schmidt and Jordan 2000). One hypothesis is that activation of serotonergic systems reconfigures spinal neuronal networks into various functional “states” (Hochman et al. 2001). For example, serotonin (5-HT$_2$) receptor activation facilitates flexion reflexes (Machacek et al. 2000) and a “state” of facilitated flexion withdrawal reflexes could serve to protect damaged tissue from further injury. Most studies have demonstrated that spinal 5-HT$_2$ receptors mediate increases in spinal cord excitability. 5-HT$_2$ receptors depolarize dorsal horn neurons in frog (Tan and Miletic 1992) and facilitate focal stimulation-induced synaptic activity in ventral horn cells (Yamazaki et al. 1992). 5-HT$_2$ receptor activity also depolarizes (Holohan et al. 1990; Wang and Dun 1990) and increases glutamate-evoked firing in motoneurons (Jackson and White 1990) and facilitates extensor stretch reflex and tonic motor activity (Miller et al. 1996). In addition, 5-HT$_2$ receptors facilitate an L-type calcium current and thus promote plateau potentials in turtle motoneurons (Perrier and Hounsaard 2003).

Activation of monoaminergic metabotropic receptors by descending neuromodulatory commands can also produce long-lasting changes in spinal cord function (Hori et al. 1996; Lewis et al. 1993). For example, 5-HT$_2$ receptors mediate a long-lasting facilitation of miniature excitatory postsynaptic currents in superficial dorsal horn neurons (Hori et al. 1996). Also in dorsal horn, low concentrations of serotonin and selective 5-HT$_2$ agonists induce a lasting synaptic enhancement at least in part as a result of recruitment of previously silent synapses (Li and Zhuo 1998; Li et al. 1999). 5-HT$_2$ receptors are also involved in long-term facilitation both of postsynaptic responses in neonatal rat hypoglossal motoneurons (Bocchiaro and Feldman 2004) and of adult respiratory motor output (Baker-Herman and Mitchell 2002).

Although 5-HT has an overall depressive effect on spinal reflexes (Crick and Wallis 1991; Hedo and Lopez-Garcia 2002; Hedo et al. 2002; Machacek et al. 2001), we previously demonstrated that the washout of 5-HT or 5-HT$_{2C}$ receptor agonists produces long-lasting facilitation of monosynaptic and longer-latency reflexes (Machacek et al. 2001). Although an increased monosynaptic reflex may in part be explained by altered activity in muscle spindle afferents, the afferents mediating longer-latency reflex facilitation remain unknown. Central sensitization, for example, is a long-lasting facilitation in spinal cord nocisponsive activity mediated by A$\delta$ and C fiber nociceptive input (Coderre et al. 1993) and facilitated nociceptive afferent responses may be responsible for our previously observed amplification in longer-latency reflexes (Machacek et al. 2001).

The experiments described in the present study explore the spinal network properties and modulatory mechanisms that produce long-lasting alterations in spinal cord reflex excitability even after the washout of the receptor agonists. We examined whether the expression of LLFR was dependent on the coincident activation of reflex pathways during 5-HT receptor activation, on GABAergic and glycnergic inhibitory actions,
and characterized the ionotropic glutamate receptors required for the expression of LLFR. In addition to understanding properties of 5-HT$_2$ receptor–induced LLFR, we also identified a 5-HT$_1A$ receptor–induced long-lasting depression of reflexes (LLDR) and demonstrate that lasting facilitation and depression are interconvertible.

**METHODS**

**Dissection**

Neonatal-juvenile Sprague–Dawley rat pups of both sexes, age range P2–P17 (but primarily P8–P14) were anesthetized with 10% wt/vol urethane (1.5–2.0 g/kg injected intraperitoneally). The animals were then submerged in an ice slurry bath for 5 min to reduce body temperature. One of two Na$^+$ substitution solutions were used during the surgical isolation of the spinal cord: 1) a high sucrose-containing artificial cerebral spinal fluid (aCSF) [(in mM): sucrose 250; KCl 2.5; NaHCO$_3$ 26; NaH$_2$PO$_4$ 1.25; d-glucose 25; MgCl$_2$ 3; CaCl$_2$ 1; and kynurenic acid 1] or 2) a high choline-containing solution [(in mM): choline chloride 110; KCl 2.5; NaH$_2$PO$_4$ 1.2; Na-pyruvate 2.4; t-aspartic acid 1.3; dextrose 20; CaCl$_2$ 0.5; MgCl$_2$ 7] (Hoffman and Johnston 1998). The animals were decapitated and the cervical to sacral spinal cord was isolated through a ventral approach. Vertebral bodies were removed with special care to maintain both ventral and dorsal spinal roots. After the dura was removed the whole spinal cord was pinned ventral side up in a Sylgard-coated (Dow) petri dish. Sagittal hemisection of the cord from C7–S1 was performed using insect pins (1.0 mm, Fine Science Tools) and each hemisection was transferred to a separate dish in oxygenated aCSF containing (in mM): NaCl 128; KCl 1.9; D-glucose 10; MgSO$_4$ 1.3; CaCl$_2$ 2.4; KH$_2$PO$_4$ 1.2; and NaHCO$_3$ 26. Ventral and dorsal lumbar roots were pinned down to the coated dish at the most peripheral cut ends. The preparation was then allowed to recover for about 1 h before any further manipulation.

**Experimental setup**

Bipolar glass suction electrodes were attached to dorsal roots L2 and L5 to allow for electrical stimulation as well as L2 and L5 ventral roots to record evoked reflexes (Fig. 1A). In some experiments, an additional suction electrode was attached to the L5 dorsal root to record afferent volleys. Constant current stimulators (Eide 1972) delivered single-shock stimuli of defined stimulation parameters to the dorsal roots. Although the present work sought to examine modulatory action on reflexes evoked by low-threshold nonnociceptive afferents, initial experiments delivered stimuli of 50 $\mu$A and 50 $\mu$s, 500 $\mu$A and 50 $\mu$s, and 500 $\mu$A and 500 $\mu$s, which roughly correspond to progressive recruitment of Aβ, Aδ, and C afferent fibers, respectively (Thompson et al. 1990). In several experiments, L2 and L5 dorsal roots were stimulated separately with an 800-ms delay between stimulation of L2 and L5 while recording reflexes from L2 and L5 ventral roots. Raw data were collected with Clampex software (v. 7.0, Axon Instruments, Union City, CA) and stored in a PC for off-line analysis. Reflex responses were collected after dorsal root stimulation frequencies of 0.02–0.1 Hz. In the majority of experiments, after an initial stabilization period, no time-dependent changes in reflex amplitude were observed at these frequencies. We used a static bath preparation that was directly oxygenated with a gas mixture of 95% O$_2$–5% CO$_2$.

**Measurement of the reflex**

To allow for quantitative analysis, reflex responses were rectified and integrated. The rectified/integrated reflex amplitude (area under the curve) was compared between baseline, drug, and wash conditions. Scatterplots indicate the area under the curve of the reflex as a

**FIG. 1.** Serotonin (5-HT) is capable of inducing long-lasting facilitation of reflexes (LLFR) and long-lasting depression of reflexes (LLDR) but selective 5-HT$_2A/C$ receptor activation with 1-(2,5)-dimethoxy-4-iodoamphetamine (DOI) induces only LLFR. A: experimental setup. Drawing of a midsagittally hemisected rat spinal cord. Glass suction electrodes are attached to dorsal roots for stimulation and ventral roots for recording of reflexes. In addition, in some experiments, a recording electrode was also attached to the L5 dorsal root to record afferent fiber volleys. B and C: scatterplots of changes in reflex strength in 2 different animals after application of 5-HT and its removal. In B, LLFR is induced whereas in C, 5-HT induces LLDR. D: effect of DOI on the sample population ($n = 34$). Values are presented as reflex strength normalized to the mean amplitude value of reflexes evoked in the 30-min period before DOI application. This period was always used to define baseline. Mean reflex values were first obtained for a given animal over 10-min intervals and were used to calculate the population mean ± SE.

whole (i.e., both early and later latency components) measured to 100 ms after the stimulus artifact beginning at the onset of the reflex response. Because of our interest in plasticity of Aβ afferent fiber-evoked reflexes this latency period was chosen to largely exclude a contribution from nociceptive afferents. Hedo et al. (1999) demonstrated in this preparation that C fiber–mediated reflexes begin at a minimal latency of 100 ms. In some cases we divided the early reflex into short- versus longer-latency responses. Short-latency, presumably...
monosynaptic, components were defined as from the onset of reflex activity to 15 ms after the stimulus artifact and longer-latency components were measured from 15 to 100 ms after the stimulus artifact (Crick and Wallis 1991). Unless otherwise stated all measured reflexes combine both short- and longer-latency components (≤100 ms after stimulus artifact).

**Electrophysiology**

We examined the stability of reflex amplitude over time under control conditions that included different stimulation frequencies (0.1 Hz, n = 5; 0.05 Hz, n = 4; and 0.033 Hz, n = 7). In several instances, reflex amplitude decayed in the first 10–30 min, but then stabilized and remained stable even after several solution exchanges or rest periods of 10 min (n = 12/16). Occasionally, the wash procedure produced slight but visible mechanical perturbations of the preparation and commonly caused a short period of facilitated reflex responses (about 5–15 min), perhaps as a result of transient activation of mechanosensitive events (Grillner et al. 1984). Alternatively, the wash procedure could enhance the diffusion of oxygen into the tissue and at the same time wash out lactate and other metabolites. This could transiently facilitate the reflex activity. However, control experiments where this procedure was repeated several times showed that the reflex returned to baseline amplitude in nine of ten experiments. In the tenth experiment the shorter-latency component continued to be facilitated after the bath solution exchange. Thus we waited until a steady reflex amplitude was obtained and define this amplitude as our control baseline (between 30 and 90 min).

Reflexes were monitored in the same way throughout the experiments. For the majority of experiments, dorsal root stimulation parameters were 500 μA, 500 μs, at 0.03 Hz unless otherwise indicated. Only preparations that expressed a steady control baseline were used for analysis.

**Pharmacology**

Stock solutions of drugs (10–100 mM) were made and stored at −20°C until needed. Bath application reflects final concentrations. Drug application involved applying stock solutions of test drugs dissolved in the appropriate solvent (aCSF, water, dimethyl sulfoxide [DMSO], or ethanol) mixed directly into the bath. A static bath was used to minimize the overall volume of drugs used in experiments. Solution exchange was achieved by replacing the bath solution three times. The neurochemicals and concentrations used were as follows: serotonin (5-HT, 10 μM); 6-chloro-2-(1-piperazinyl) pyrazine (MK212, 1 μM); 1-(2,5)-dimethoxy-4-iodoamphetamine (DOI, 1–10 μM); 8-hydroxy-2-(dipropylamino)-tetralin (8-OH-DPAT, 1 μM); clozapine (0.5–2 μM); kynurenic acid (1 mM); bicuculline methiodide (10–20 μM); phenylbenzene-α-phosphono-α-amino acid (PMBA, 50–100 μM); d-α-amino-5-phosphonovoronic acid (AP5, 50 μM), and 6-cyano-7-nitroxiquinoxaline-2,3-dione (CNQX, 1–10 μM). All drugs were obtained from Sigma-RBI.

**Immunohistochemistry**

Six male Sprague–Dawley rats (Raleigh, NC) aged P14 were perfused with a solution containing 0.9% saline, 0.1% sodium nitrate, and 0.01% heparin followed by Lana’s fixative [4% paraformaldehyde, 0.2% picric acid, 0.16 M phosphate (PO4) buffer (pH 6.9)]. Spinal cords were isolated, postfixed for 2 h in 4% paraformaldehyde, and cryoprotected in 10% sucrose with 0.1 M phosphate (PO4) then stored at 4°C. Lumbar spinal cords were frozen at −80°C, then sectioned in 10-μm-thick slices on a cryostat and collected on microscope slides. Slides were rehydrated for 4 h in 1 M PO4-buffered saline (PBS) at room temperature (RT). They were then washed overnight in PBS containing 0.3% Triton X-100 (PBS-t) at 4°C. Tissue was incubated in mouse anti 5-HT2A (diluted 1:500, Pharmingen) or mouse anti 5-HT1C (diluted 1:250, Pharmingen) for 48–72 h at 4°C, then washed three times in PBS-t for 30 min each time at RT. Tissue was then incubated in biotinylated donkey anti-mouse (diluted 1:250, Jackson Labs) for 1.5 h at RT and then washed three times for 20 min each in PBS-t. After this, tissue was incubated in extravidin-Cy3 (Sigma) diluted 1:1,000 for 1.5 h, washed once for 20 min in PBS-t, followed by washing two times for 20 min each in 50 mM Tris–HCl. Finally, slides were coverslipped with Vectashield (Vector Labs) and photographed with either a Nikon E-800 Microscope using a DXM-1200 Digital Camera or scanned using a BioRad MRC-60 confocal microscope on a Zeiss axiovert-135 inverted microscope.

**Data analysis**

In determining differences between control and drug conditions, the evoked responses from the last 10 min of each condition were compared to ensure that the full drug effect was measured. Population means were determined as a percentage change compared with the control condition. Figures typically show an example of the response as well as the population histograms. Values are given as means ± SE. LLFR and LLDR were considered to have occurred when the area under the curve of the rectified responses remained significantly different from the control baseline reflex response for ≥1 h after washout (SigmaPlot and SigmaStat; SPSS Science, Chicago, IL). Normality was tested (P ≤ 0.05) and paired t-test or ANOVAs were used unless normality failed, in which case nonparametric tests were used. All differences were considered significant if P < 0.05. There was no statistical difference in the degree of facilitation after washout of DOI when comparing hemisects obtained from the same animal to those obtained from different animals (P ≤ 0.05, n ≥ 12) and thus the data from all hemicords were pooled together. Unless otherwise stated, experiments were conducted on the Sprague–Dawley rat sub-strain obtained from North Carolina.

**RESULTS**

The purpose of these experiments was to characterize the actions of 5-HT receptor activation on long-lasting changes in the input–output properties of spinal segmental reflexes. The sample population consisted of 186 hemisected spinal cords obtained from 136 animals. Control experiments were run to define the general properties of afferent fiber recruitment as well as to test the variability of evoked spinal reflexes elicited by electrical stimulation of afferents in the dorsal roots. Two suction electrodes, one for stimulating and one for recording, were placed on the same dorsal root (usually L5) at a mean distance of approximately 4 mm apart. It was previously reported that electrical stimulation of peripheral nerves progressively recruited Aβ, Aδ, and C fiber volleys with stimulus parameters of 50 μA and 50 μs, 500 μA and 50 μs, and 500 μA and 500 μs, respectively (Thompson et al. 1990). Although this was also commonly observed in our sample with electrical stimulation of dorsal roots, in many animals C fibers could also be recruited at intensities lower than 500 μA and 500 μs but never at an intensity of 50 μA and 50 μs. Therefore although we did not determine stimulation as a multiple of threshold for each experiment we are confident that we were not recruiting C fibers at 50 μA and 50 μs. Afferent conduction velocity, estimated from onset of stimulus artifact to the first peak negativity of the triphasic volley, was 2.3 and 0.24 m/s, respectively, for the fastest arriving A fibers and C fiber components. The C fiber conduction velocity is consistent with that previously reported in vitro (Hedo et al. 1999; Lu and Perl 2003). The conduction velocity of Aβ fibers recorded here is
similar to but lower than that observed in sciatic nerve recordings in similar aged rats (8 m/s) at RT and much slower than that observed in adult as reported at 36°C (Aβ = 22.4 m/s, Aδ = 8.0 m/s) (Hedo et al. 1999; Park et al. 1999). We assume these differences are temperature dependent.

5-HT receptor–mediated long-lasting changes in reflex strength: substrate comparison

Reflexes were recorded in ventral roots after dorsal root stimulation (typically L5; Fig. 1A). Previously, we observed that after washout of bath-applied 5-HT, long-lasting facilitation of reflexes (LLFR) was observed in 13/14 Sprague–Dawley rats (Machacek et al. 2001). The animals in that study were obtained from the Charles River colony in Montreal, Canada. The present series of experiments began with Sprague–Dawley rats obtained from the Charles River colony in Raleigh, NC. In this population, after 5-HT washout, LLFR was observed in only four of 13 cases (Fig. 1B), whereas a novel observation, a long-lasting depression of reflexes (LLDR), occurred in six of 13 animals (Fig. 1C). Whereas the present report focuses on aspects of 5-HT2 receptor–induced LLFR, the observation that LLDR as well as LLFR can be observed from this colony demonstrates that reflex gain can be modulated upward and downward, affording a bidirectional characterization of 5-HT receptor–induced plasticity in reflex strength (described later).

One explanation for the observed differences in reflex plasticity between the present and previous work is substrate differences. To confirm this, we retested the Sprague–Dawley rats from Montreal and observed a robust LLFR after 5-HT washout in four of five experiments (not illustrated). Additional differences in reflex excitability and plasticity in these substrains are reported below and summarized in Table 1.

DOI is a potent nonselective 5-HT2A/2C receptor agonist that we previously reported to induce LLFR in five of five animals (36). Figure 1D depicts the time course of DOI-induced LLFR for the present sample population (n = 34) where LLFR was observed in 31/34 hemicords. Unlike the observation with 5-HT, the ability of DOI to induce LLFR appeared to be equally effective for both substrains, producing LLFR in 15/17 from Raleigh and 16/17 from Montreal (Table 1; 3rd data column).

We next compared the effects of DOI on both substrains at two constant-current stimulus intensities that recruit a subpopulation of Aβ fibers and fully recruit all afferents (Fig. 2, A and B). In comparison to the Raleigh colony, spinal cord reflex excitability in the Montreal colony was significantly greater by several measures (Table 1). For example, at stimulus strength of 50 μA and 50 μs, which usually recruited only a small portion of the Aβ fiber volley, the Montreal colony has a greater incidence of evoked short- and long-latency reflexes both before and after DOI application. Because the long-latency reflexes were observed when only a small component of the fastest DR volley was recruited, these actions must be mediated by Aβ afferents. Interestingly, after DOI, in 27 and 33% of the Montreal animals, short- and long-latency reflexes were unmasked, respectively, at a stimulation intensity that recruits only a small portion of Aβ fibers (see Fig. 2B, left). Overall, comparing the two populations, in the Montreal colony, reflexes are more easily evoked and Aβ fiber–mediated reflexes are also more likely to be facilitated after 5-HT2 receptor activation (Table 1).

Afferent fibers involved in LLFR

We next compared different stimulation intensities in relation to the DOI-induced LLFR in animals in which we recorded afferent fiber volley. In 11/11 animals, recruitment of a small fraction of Aβ fibers alone (at 50 μA and 50 μs) produced a reflex that was susceptible to DOI-induced LLFR (Fig. 2, A and B, left). Indeed, even when much greater stimuli were delivered that maximally recruit Aβ fibers and also recruit Aδ and C fibers (Fig. 2, A and B, right) the early-latency–facilitated reflex amplitude was not substantially greater (examined in nine animals at 500 μA and 500 μs). Thus within the reflex period examined, the reflex facilitated by DOI is mediated predominantly by Aβ fibers. Therefore to ensure maximal recruitment of Aβ fibers, all of the following experiments were conducted at a stimulation intensity of 500 μA and 500 μs. In 31 animals with DOI-induced LLFR, long-latency components were always observed and were facilitated. Short-latency monosynaptic reflexes were observed in 27/31 of these animals, 25/27 of which were facilitated by DOI. In the four animals initially without monosynaptic reflexes, short-latency reflexes emerged in three animals after DOI. Thus the predominantly Aβ-mediated short- and longer-latency reflexes appear to be equally facilitated. Thus for the subsequent experiments where reflexes are quantified, monosynaptic and longer-latency actions are grouped together (the first 100 ms of the reflex after the stimulation artifact).

Heterosegmental actions between L2 and L5 spinal segments

In eight animals both L5 and L2 dorsal roots were stimulated at 500 μA and 500 μs (800-ms delay between stimuli) while simultaneously recording from L5 and L2 ventral roots. Before

<table>
<thead>
<tr>
<th>Table 1. Comparison of 5-HT- and DOI actions on spinal reflexes between Montreal and Raleigh colonies of Charles River Sprague–Dawley rats</th>
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<tbody>
<tr>
<td><strong>Colony</strong></td>
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<tr>
<td>Montreal</td>
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<tr>
<td>Raleigh</td>
</tr>
<tr>
<td><strong>Occurrence of Evoked Reflexes at 50 μA and 50 μs</strong></td>
</tr>
<tr>
<td>Montreal</td>
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<tr>
<td>Raleigh</td>
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Facilitated reflexes are not the result of activity-dependent homosynaptic strengthening

Previously, reflexes were obtained using stimulation of dorsal root afferents at low frequency (0.02 Hz) (Machacek et al. 2001). At this frequency, it may be suggested that the 5-HT-induced changes are independent of stimulation-evoked intrinsic network synaptic activity. In the present study, to further minimize a contribution from electrically evoked reflex recruitment to the observed plasticity, only three to five stimuli were delivered per hour to capture reflex strength before, during, and after DOI application (Fig. 2, A and B). Under these conditions, marked (62%) increase and long-lasting increases in reflex strength were still observed (n = 18/19; 14/15 Montreal colony and 4/4 Raleigh colony). Thus the induction of DOI-induced alterations in reflex strength appears to occur by mechanisms dependent on 5-HT receptor activation, but independent of coincident activity of the reflex circuit.

Spinal distribution of 5-HT_{2C} and 5-HT_{2A} receptors

Although pharmacological studies clearly suggest that LLFR can be induced by 5-HT_{2C} receptor activation for its induction (Machacek et al. 2001) a contribution from 5-HT_{2A} receptors cannot be ruled out. For example, the most robust LLFR is evoked after washout of DOI, a potent 5-HT_{2A/2C} agonist. Therefore using immunocytochemistry, we compared the distribution of 5-HT_{2C} and 5-HT_{2A} receptors in the lumbar spinal cord of six P14 rats. Figure 3 provides a representative example of the labeling pattern observed. Note that 5-HT_{2C} receptor labeling appears to predominate in the gray matter, with greatest labeling density in the deep dorsal horn, intermediate gray matter, and motor nuclei. The labeling is punctate, intense, and includes perisomatic labeling (Fig. 3A). In comparison, 5-HT_{2A} receptor labeling is dominant in the white matter and is not clearly juxtaposed to neuronal cell somas (Fig. 3B), although labeling in some neurons cannot be ruled out. Thus the actions of DOI are likely to be by 5-HT_{2C} receptor activation. The prominence of 5-HT_{2C} receptor labeling in the intermediate gray matter and ventral horn coincides with the location of many interneurons and motoneurons associated with the coordination of motor activity (Baldissera et al. 1981; Goulding and Pfaff 2005; Jankowska 1992, 2001). In addition, AB fibers terminate in these regions (Willis Jr and Coggeshall 1991), consistent with our electrophysiologic observations of an effect predominantly mediated by low-threshold afferents.

Effects of GABA_{A} and glycine receptor antagonists

The observed increases in reflex strength could result from a reduction in the actions of inhibitory interneurons, either interposed in reflex pathways or producing a presynaptic inhibition at excitatory synapses. Thus we tested the contribution of inhibitory interneurons to the evoked plasticity. Instead of DOI we chose to examine the actions of the selective but partial 5-HT_{2C} receptor agonist MK212 (Porter et al. 1999). MK212, a weak partial agonist, was chosen instead of DOI in these experiments because the occurrence and strength of the evoked LLFR is much smaller (see Table 2) and thus should provide a better model to examine the contributory effects of a disinhibited spinal cord. Spinal reflexes were examined in 11 experi-
antagonist (Hosie et al. 1999). The more commonly used glycine receptor antagonist strychnine was not used because it produced irreversible increases is spinal reflex excitability, probably arising from its membrane permeance and consequent block of various ion channels (Lee et al. 1975; Shapiro 1977). However, experiments with PMBA suggested that it might be blocking MK212 actions on the 5-HT2 receptor. To overcome this, we applied the full agonist DOI at 10 μM, and under these conditions, LLFR was observed in four of five instances in the presence of glycine receptor blockade (Fig. 4C). Further, when both GABA<sub>A</sub> and glycine receptors were blocked simultaneously, DOI induced LLFR in five of five cases (Fig. 4D). Thus neither GABA<sub>A</sub> nor glycine receptor activity is required for the induction of 5-HT<sub>2</sub> receptor–mediated increases in reflex strength.

**Effects of NMDA and non-NMDA receptor antagonists**

We next studied whether 5-HT<sub>2A/2C</sub> receptor–induced LLFR requires activation of N-methyl-d-aspartate (NMDA) and non-NMDA ionotropic glutamate receptors. Activation of 5-HT<sub>2A/2C</sub> receptors with DOI in the presence of the NMDA receptor antagonist AP5 induced LLFR in three of three cases (Fig. 5A). In other experiments we preincubated the spinal cord in bicuculline to better isolate reflex modulation resulting from alterations in glutamatergic synaptic activity (Fig. 5B). Subsequent addition of AP5 depressed longer-latency reflexes in 11/12 cases while leaving the short-latency reflex component largely unchanged (not shown). Comparison of reflex responses in the presence of AP5/bicuculline before and after activation of 5-HT<sub>2</sub> receptors with MK212 or DOI revealed LLRF in seven of 12 cases. Thus 5-HT<sub>2</sub> receptor activation can potentiate non-NMDA receptor reflex components independent of NMDA receptor activity and this facilitation cannot be based entirely on a reduction in tonic GABA<sub>A</sub> receptor activity (Fig. 5B).

Experiments were performed where DOI was added in the presence of the α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA)/kainate receptor antagonist CNQX. In five of five cases CNQX depressed both short- and reduced-latency reflex components. Addition of DOI did not alter the reflex blocking actions of CNQX and after removal of all drugs, reflexes reemerged slowly, generally returning to control values. Even when inhibitory input was blocked with bicuculline, addition of 1 μM DOI in the presence of CNQX (10 μM) failed to cause LLFR (n = 3). Interestingly, when a


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**FIG. 3.** 5HT<sub>2C</sub> and 5-HT<sub>2A</sub> receptor labeling in lumbar spinal cord of a P14 rat. For both 5-HT<sub>2C</sub> (A) and 5-HT<sub>2A</sub> receptors (B), left panel presents low power photomicrograph of one side of a spinal cord transverse section (scale bar = 100 μm) and right panels are confocal images displaying labeling at higher magnification (scale bar = 25 μm) of superficial dorsal horn (laminae I–II) and ventral horn lamina IX (top and bottom, respectively). Images are presented as grayscale negatives. Drawn white lines approximate white matter/gray matter border and major divisions of Rexed’s laminae. A: 5-HT<sub>2C</sub> receptor labeling is strongly expressed in the deep dorsal horn (lamina III–VI) intermediate lamina VII and motor nuclei (lamina IX). Arrows in right panels point to cell somas with perisomatic punctate labeling in dorsal horn (top) and lamina IX neurons in ventral horn (bottom). B: 5-HT<sub>2A</sub> receptor labeling is strongest in the white matter. Note that labeling in the gray matter is uniformly weak. Arrows point to punctate labeling that is not clearly juxtaposed to cell somata in the superficial dorsal horn (laminae I–II) and in the motor nucleus (lamina IX).

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**TABLE 2. Summary of data for all conditions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percentage Change From Control</th>
<th>n</th>
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<tbody>
<tr>
<td>DOI</td>
<td>162 ± 14</td>
<td>31/34</td>
</tr>
<tr>
<td>MK212 + bicuculline</td>
<td>181 ± 42</td>
<td>4/5</td>
</tr>
<tr>
<td>DOI + AP5</td>
<td>148 ± 8</td>
<td>3/3</td>
</tr>
<tr>
<td>DOI + CNQX</td>
<td>141 ± 16</td>
<td>3/3</td>
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<tr>
<td>DOI + kynurenate</td>
<td>179 ± 24</td>
<td>4/4</td>
</tr>
<tr>
<td>DOI + PMBA</td>
<td>142 ± 21</td>
<td>4/5</td>
</tr>
<tr>
<td>DOI + bicuculline + PMBA</td>
<td>187 ± 58</td>
<td>5/5</td>
</tr>
<tr>
<td>MK212 or DOI + bicuculline + AP5</td>
<td>140 ± 27</td>
<td>7/12</td>
</tr>
<tr>
<td>MK212</td>
<td>106 ± 10</td>
<td>6/11</td>
</tr>
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Values are means ± SE; n represents the fraction of total cords showing long-lasting facilitation of the reflex, P < 0.05.
higher concentration of DOI (10 μM) was used, LLFR emerged (three of three; Fig. 5C). Because increased concentrations of DOI overcome the CNQX effect, it is possible that CNQX blocks 5-HT2 receptors as well as its commonly accepted block of AMPA/kainate receptors. In support of this, in the presence of NMDA and AMPA/kainate the receptor antagonist kynurenic acid (1 mM), which completely blocked reflex activity, 1 μM DOI caused a robust LLFR after its washout (four of four; Fig. 5D). In total, these results suggest that facilitated responses include contributions from both NMDA and AMPA/kainate receptors but that neither is required for the induction of LLFR.

Interconvertibility of LLFR and LLDR by receptor-selective activation

Although 5-HT2 receptors generally facilitate spinal neural responsiveness, 5-HT1 receptor activation usually depresses neural activity (see Hochman et al. 2001). Thus we tested whether 5-HT2-mediated LLFR could be reversed with activation of 5-HT1 receptors and vice versa. Interestingly, when 5-HT1A receptors were selectively activated with the 5-HT1A receptor agonist DPAT (in the presence of clozapine to block DPAT’s actions at 5-HT7 receptors) the 5-HT2/3/2c receptor-mediated LLFR was partly reversed in seven of nine cases (six of nine significantly; Fig. 6A). Conversely, 5-HT1A receptor activation induced a long-lasting reflex depression (LLDR; four of six) that was reversed with subsequent 5-HT2/2c receptor activation (six of six), and even increased reflex strength to LLFR (wash 2 compared with wash 1) in four of six instances (Fig. 6B). Thus reflex strength can be modulated bidirectionally depending on the 5-HT receptor subtype activated.

**DISCUSSION**

We observed that transient 5-HT2 receptor activation in the juvenile isolated rat spinal cord can result in enhanced reflex strength that is maintained for ≥1 h. Although not explored here, LLFR could be observed even 12 h after its induction (data not shown). Reflex facilitation commonly required removal of the agonist for expression. After washout, 5-HT2/2c receptor–mediated LLFR was produced regardless of whether there is concurrent antagonism of ionotopic excitatory glutamatergic and/or inhibitory GABAergic/glycinergic receptor activity. Therefore although the reflexes themselves appear to be mediated by glutamate, induction and maintenance of LLFR are independent of classic, fast transmission ionotopic receptor activity. 5-HT2/2c receptor–induced LLFR also appeared to be independent of other mechanisms associated with afferent-evoked activity because it was observed when measured with as few as three stimuli per hour. The magnitude of the induced LLFR was limited by GABAergic actions depending on the 5-HT receptor subtype activated.

An important additional observation was that 5-HT could also induce a long-lasting depression of reflexes (LLDR), mimicked by activation of 5-HT1A receptors, and that LLFR and LLDR are interconvertible with 5-HT2/2c and 5-HT1A receptor agonists, respectively. Therefore the serotonergic system has the capacity to induce long-lasting bidirectional changes in reflex strength.
Facilitatory effects of serotonin in spinal cord

Serotonergic neurons are not intrinsic to the spinal cord so modulatory actions normally arise from activity in several descending bulbospinal systems (e.g., raphe nuclei; see Hochman 2001 for review; however, see Newton and Hamill 1988).

Both facilitatory and depressant actions have been reported with a heuristically attractive but oversimplified scheme, suggesting that 5-HT inhibits sensory and promotes motor activity (Hochman et al. 2001; Jacobs and Fornal 1993). Based on pharmacological evidence in an earlier study, we determined that 5-HT produced a lasting reflex amplification by activation of 5-HT$_{2C}$ receptors (Machacek et al. 2001). Immunohistochemical analysis of 5-HT$_{2C}$ receptor distribution here indicates a relative lack of receptors in the superficial dorsal horn (lamina I and II) with much greater labeling in deeper spinal laminae (Fig. 3). Because the majority of nociception-encoding C fibers terminate in laminae I and II (Willis Jr and Coggeshall 1991), one would expect that the 5-HT$_2$ receptor–induced reflex facilitation does not primarily involve nociceptive afferent input. Indeed, the 5-HT$_{2A/2C}$ receptor–induced facilitation studied here was primarily mediated by A$_B$, low-threshold afferent fibers, which project to 5-HT$_{2C}$ receptor–rich deeper spinal laminae. 5-HT$_{2C}$ receptors are thought to be postsynaptic on the somatodendritic membrane (Leyesen 2004), consistent with the presently observed spinal perisomatic labeling.

Irrespective of the substrain differences observed, both the short- and longer-latency (presumably polysynaptic) reflex responses appear to be facilitated by 5-HT$_{2A/2C}$ receptor activation after agonist washout. Because in some instances only the longer-latency reflex component was facilitated, whereas the monosynaptic reflex was unchanged, the modulation of spinal cord reflex excitability is unlikely to simply reflect changes in motoneuron properties (Bennett et al. 1998; Carlin et al. 2000; Lee and Heckman 1998; Perrier and Hounsgaard 2003). Indeed, the emergence of long-latency heterosynaptic actions between L2 and L5 spinal segments after 5-HT$_{2A/2C}$ receptor activation support an increased excitability through interneuronal pathways. Although some heterosynaptic facilitatory actions could be caused simply by a lasting postsynaptic motoneuronal depolarization, previous recordings demonstrate a corresponding facilitated convergent input onto interneurons from distant spinal segments (Machacek et al. 2001). Moreover, unlike the long-lasting actions currently described, previous in vitro studies on motoneuron properties demonstrated that the actions of 5-HT or 5-HT$_2$ receptor agonists recover after agonist removal (Berger and Takahashi 1990; Elliott and Wallis 1992; Elliott et al. 1999; Takahashi and Berger 1990; Wang and Dun 1990). The involvement of interneurons in increased reflex excitability was also suggested by observing long-lasting increases in excitatory postsynaptic potential (EPSP) amplitudes in a subpopulation of laminae IV–VII neurons (Machacek et al. 2001). Moreover, complementary studies in cat have demonstrated that polysynaptic flexion and crossed-extension reflexes are facilitated by DOI, and in a temporally distinct manner from facilitated stretch reflexes (Machacek et al. 2000). Thus the heterosegmental facilitation of reflexes observed here is consistent with spinal plasticity at the interneuronal level. Intracellular recordings from motoneurons and interneurons will be required to better clarify the relative contribution of pre- versus postsynaptic mechanisms to the production of LLFR. Because the 5-HT$_2$ receptor mechanisms inducing reflex plasticity were observed to be independent of activity in the afferent fibers reporting LLFR, plasticity occurs by nonassociative mechanisms. In a similar manner, 5-HT$_3$ receptors have been reported to be involved in long-lasting facilitated postsyn-
aptic responses in neonatal rat hypoglossal motoneurons by activity-independent mechanisms (Bocchiaro and Feldman 2004). 5-HT2 receptor activity may also be responsible for facilitation of other synaptic responses in spinal cord independent of synaptic activation. For example, 5-HT2 receptor activation has been found to be involved in activating protein kinase C (PKC) and phosphorylating NMDA receptors, thereby potentiating NMDA receptor–mediated ion currents (Blank et al. 1996) and activation of PKC, which has been implicated in persistent pain and hyperalgesia (Wajima et al. 2000; Yashpal et al. 1995).

Effects of GABA_A and glycineergic disinhibition

LLFR was observed after blockade of GABA_A and/or glycine receptors. This demonstrated that alterations in inhibitory mechanisms are not required to induce or maintain LLFR. Also, because the strength of the evoked LLFR was much greater when GABA_A receptors were blocked, factors associated with GABAergic inhibition appear to be able to limit LLFR strength. Although not explored it is possible that 5-HT2 receptor activation also facilitates transmission through inhibitory pathways.

Because temporary increases in network excitability, such as with GABA_A or glycine receptor block (bicuculline and PMBA, respectively), could predispose activated synapses toward long-lasting changes in excitability, it was critical to compare 5-HT2A/C receptor LLFR in experiments where GABA_A or glycine receptor blockade was present throughout the test period. Indeed, PMBA and bicuculline on their own were observed to be capable of producing long-lasting increases in reflex strength after washout (not shown). These effects are unlikely attributable to incomplete drug washout because, in a similar preparation, these drugs are reversible with washout when examining other spinal mechanisms (Cowley and Schmidt 1995; Kremer and Lev-Tov 1997; Sajovic and Levinthal 1983).

NMDA and non-NMDA receptor–mediated facilitation

Evoked reflexes include non-NMDA (AMPA/kainate) receptor–mediated monosynaptic and NMDA and non-NMDA receptor–mediated polysynaptic reflexes (Evans 1989; King et al. 1992). We had previously demonstrated that both components underwent a 5-HT receptor–dependent LLFR (Machacek et al. 2001). Here, initial experiments using the quinoxalinedione CNQX to block AMPA/kainate receptors prevented DOI-induced LLFR. However, radioligand binding assays suggest that various quinoxalinediones compete for binding at 5HT2 receptors (Soskic and Joksimovic 1998). We thus increased the concentration of DOI to 10 μM to surmount a possible competitive antagonism and, under these conditions, LLFR was observed. Furthermore, kynurenic acid, a broad-spectrum ionotropic glutamate receptor antagonist that blocks both AMPA/kainate and NMDA receptors, blocked reflex activity while in the bath, but did not prevent DOI-induced LLFR after washout. Together these results clearly demonstrate that 5-HT2A/C receptor–induced facilitation occurred independently of NMDA and AMPA/kainate receptor activation.

Properties of 5-HT2 receptors and possible mechanisms producing enhanced facilitation after agonist removal

Once activated, the DOI-induced LLFR is maintained even after the addition of normethyl-clozapine (Machacek et al. 2001) to block 5HT2C receptors. Thus maintenance of LLFR is not dependent on agonist-based continued activation of 5HT2C receptors. Indeed, in the majority of experiments, reflex facilitation required removal of agonist for expression. Because 5-HT2A/C receptor–induced LLFR occurred after activation with its endogenous ligand (5-HT), the long-lasting changes do not arise from unusual properties of an exogenous agonist. Interestingly, DOI induced LLFR much more robustly than MK212 and it is known that different 5-HT2 receptor agonists can bias activation toward different signal transduction path-
ways (Berg et al. 1998). In this regard the actions of DOI appear to better approximate the actions of the endogenous ligand 5-HT. The different results obtained between DOI and MK212 may also partly be explained by the partial agonist activity of MK212 at 5-HT$_{2C}$ receptors versus the full agonist activity of DOI (Jerman et al. 2001) and an involvement of 5-HT$_{2A}$ receptors to the DOI-induced reflex enhancement cannot be ruled out (Eide and Hole 1993; Hori et al. 1996; Lewis and Coote 1993).

Although the mechanism behind increased facilitation after removal of agonist is currently unknown, the complex signaling properties of 5-HT$_{2}$ receptors are implicated (Leyesen 2004; Van Oekelen et al. 2003). The 5-HT$_{2C}$ receptor is capable of coupling to several G proteins and consequently multiple signal transduction cascades. They are: 1) phospholipase C (PLC) activation by $G_{q}$, 2) phospholipase A$_{2}$ (PLA$_{2}$) by $G_{o/o}$ associated $G_{i}$-mediated activation of extracellular signal related kinases (ERKs), 3) p38 by $G_{o12,13}$, and 4) phospholipase D activation by $G_{o13}$ and free $G_{i}$-mediated PLC activation by Gq, by G$_{q}$-mediated activation of extracellular signal related kinases (ERKs), 3) p38 by G$_{o12,13}$, and 4) phospholipase D activation by G$_{o13}$ and free G$_{i}$ (Leyesen 2004). Furthermore, 5-HT$_{2C}$ receptors can become constitutively active (Grotewiel and Sanders-Bush 1999). Although these complex properties may help explain the unusual properties of facilitation after agonist unbinding, further studies are necessary to identify the cellular mechanisms involved.

**Modulation of reflex strength is bidirectional**

To be behaviorally relevant the observed lasting changes in reflex strength should be bidirectional to maintain reflex pathway operation within a normal range of sensorimotor gain. Therefore an important observation was that 5-HT can also induce a long-lasting reflex depression (LLDR). Moreover, pharmacological studies demonstrated that LLFR and LLDR are interconvertible by 5-HT$_{2A/C}$ and 5-HT$_{1A}$ receptor activation, respectively. Therefore the serotonergic system has the demonstrated capacity to induce long-lasting bidirectional changes in reflex strength. In these experiments, the facilitatory actions produced by 5-HT$_{2A/C}$ receptor activation were stronger than the depressant actions produced by activation of 5-HT$_{1A}$ receptors. Even though the 5-HT receptor pharmacology responsible for LLFR has been clearly determined to be by 5-HT$_{2C}$ receptor activation (Machacek et al. 2001), we have yet to thoroughly investigate the 5-HT receptors responsible for LLDR. Therefore it is possible that other 5-HT receptors are capable of producing LLDR or acting in a complementary manner with 5-HT$_{1A}$ receptors to produce a more profound depression. It is also possible that LLDR is more strongly induced when spinal cord reflexes are already in a facilitated state. These observations warrant further study.

Overall, these experiments suggest that serotonergic systems can be used to produce lasting changes in reflex gain. These changes appear to be able to act like a slow switch to either increase or decrease reflex excitability. We have not attempted to determine the temporal limits of the 5-HT receptor activation required to induce reflex plasticity. However, a reflex plasticity that requires agonist activity for several minutes would be expected to contribute to long-lasting transitions in excitability as occurs in the sleep/wake cycle, but would not be suited to transient excitability shifts as observed in changing postural and locomotor activities.


