Facilitatory Actions of Serotonin Type 3 Receptors on GABAergic Inhibitory Synaptic Transmission in the Spinal Superficial Dorsal Horn

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Fukushima T, Ohtsubo T, Tsuda M, Yanagawa Y, Hori Y. Facilitatory actions of serotonin type 3 receptors on GABAergic inhibitory synaptic transmission in the spinal superficial dorsal horn. J Neurophysiol 102: 1459–1471, 2009. First published April 15, 2009; doi:10.1152/jn.91160.2008. Analgesic effects of serotonin (5-hydroxytryptamine [5-HT]) type 3 (5-HT3) receptors may involve the release of γ-aminobutyric acid (GABA) in the spinal dorsal horn. However, the precise synaptic mechanisms for 5-HT3 receptors in both types of neurons by single-cell reverse transcription–polymerase chain reaction (RT-PCR) analysis. Further, GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs)—both those evoked by electrical stimulation and those occurring spontaneously in tetrodotoxin (i.e., miniature IPSCs [mIPSCs])—were recorded from GFP-negative neurons. 2-Me-5-HT increased the amplitude of the evoked IPSCs and the frequency of mIPSCs. The amplitude of mIPSCs was not affected by 2-Me-5-HT, suggesting that 5-HT augments GABAergic synaptic transmission via presynaptic mechanisms. The present observations indicate that 5-HT3 receptors are expressed on both somadendritic regions and presynaptic terminals of GABAergic neurons and regulate GABA_A receptor-mediated inhibitory synaptic transmission in the SDH. Taken together, these results provide a basis for the underlying mechanisms of the antinociceptive actions of 5-HT3 receptors in the spinal dorsal horn.

INTRODUCTION

Serotonin (i.e., 5-hydroxytryptamine [5-HT]) has been implicated in a wide variety of physiological processes. The diversity of actions is attributed to the existence of numerous different receptor subtypes: seven distinct classes (5-HT1 to 5-HT7) have been identified through pharmacological and molecular biological studies (Barnes and Sharp 1999; Hoyer et al. 2002; Tecott and Julius 1993). Among them, only 5-HT3 receptors are not coupled to G protein, but directly linked to nonselective cationic channels that mediate fast excitatory responses (Derkach et al. 1989; Giordano and Schultea 2004; Maricq et al. 1991; Yakel and Jackson 1988).

The 5-HT3 receptors are distributed in both the peripheral nervous system (PNS) and the CNS. In the PNS, 5-HT3 receptors occur on neurons of the sensory nervous system as well as on autonomic neurons and enteric neurons (Fozard 1984; Ireland and Tyers 1987; Kilpatrick et al. 1987). Within the CNS, these receptors are found primarily in limbic, brain stem, and spinal cord structures (Laporte et al. 1992; Morales et al. 1998; Tecott et al. 1993). In the spinal cord, 5-HT3 receptors are densely localized in the superficial dorsal horn (SDH) (Fonseca et al. 2001; Kia et al. 1995; Laporte et al. 1992; Tecott et al. 1993; Tsujiya et al. 1999), which receive nociceptive input and where it is modulated.

Intrathecal administration of 5-HT3 receptor-specific agonist 2-methyl-serotonin (2-Me-5-HT) has been shown to exert antinociceptive actions in behavioral nociceptive tests such as the formalin test (Giordano 1991; Sasaki et al. 2001) and tail flick and hot plate tests (Glaum et al. 1990; Paul et al. 2001). Electrophysiological studies recording the activities of dorsal horn neurons have indicated that antinociception is induced by the activation of 5-HT3 receptors in the SDH. For example, responses of SDH neurons to noxious stimuli are inhibited by electrical stimulation of the midbrain periaqueductal gray matter (PAG) and this inhibition is attenuated by spinal application of antagonists to 5-HT3 receptors (Cui et al. 1999; Peng and Willis 1996; Peng et al. 2001). It has also been reported that responses of SDH neurons to noxious cutaneous stimuli and locally applied N-methyl-D-aspartate (NMDA) and substance P are inhibited by iontophotically injected 5-HT3 receptor agonist (Alhaider et al. 1991). Accordingly, it has been suggested that 5-HT3 receptors located in the SDH are involved in antinociception.

 Among the neurotransmitters and neuromodulators implicated in the processing of sensory and nociceptive information, γ-aminobutyric acid (GABA) is also of importance in the SDH: GABAergic neurons are distributed in a high concentration in the SDH (Mackie et al. 2003; Makinae et al. 2000; Mitchell et al. 1993; Todd and McKenzie 1989; Todd and Spike 1993). The distribution of GABA_A receptors in the SDH has also been reported (Alvarez et al. 1996; Bohlhalter et al. 1996; Ma et al. 1993; Persohn et al. 1992; Takahashi et al. 2006; Wisden et al. 1991). With regard to the mechanisms of antinociceptive action of 5-HT3 receptors in the SDH, a number of issues remain to be clarified. One suggested mechanism is that GABA mediates 5-HT3 receptor-induced antinociception at the spinal level. For example, several behavioral analyses revealed that analgesia induced by spinal 5-HT3 receptor agonist is attenuated by GABA_A receptor antagonist bicuculline (BIC) (Alhaider et al. 1991; Giordano 1991; Giordano and...
Schultea 2004; Yang et al. 1998). Furthermore, electrophysiological investigations have shown that the descending antinoceptive actions induced by activation of PAG are blocked by BIC (Alhaider et al. 1991; Cui et al. 1999; Lin et al. 1994; Millan 2002; Peng et al. 2001). Accordingly, it is suggested that the activation of 5-HT₃ receptors on GABAergic interneurons evokes GABA release, resulting in antinoception via GABAₐ receptors (Alhaider et al. 1991; Kawamata et al. 2003; Lin et al. 1994, 1996; McGowan and Hammond 1993; Tamamoto et al. 2004).

The expression of 5-HT₃ receptors on GABAergic neurons has been shown in central brain regions such as the telencephalon (Morales and Bloom 1997; Morales et al. 1996) and the cerebral cortex (Puig et al. 2004) through immunohistochemical (IHC) studies. In these regions, electrophysiological investigations have shown that the activation of 5-HT₃ receptors depolarizes GABAergic interneurons, which in turn presynaptically facilitate GABAₐ receptor-mediated inhibitory synaptic transmission (Dorostkar and Boehm 2007; Ferezou et al. 2002; Turner et al. 2004; Zhou and Hablitz 1999). It remains to be elucidated, however, whether GABAergic interneurons in the SDH express 5-HT₃ receptors, as is the case in other regions of the CNS, and how the activation of these receptors affects GABAergic inhibitory synaptic transmission in the SDH.

Our investigation had two aims. First, we attempted to clarify whether GABAergic neurons in the SDH express functional 5-HT₃ receptors and then examined the expression of 5-HT₃ receptor messenger ribonucleic acid (mRNA) in these neurons. Second, we investigated how 5-HT₃ receptors affect GABAergic inhibitory synaptic transmission in the SDH, by analyzing the effects of agonists specific for 5-HT₃ receptors on GABAₐ receptor-mediated inhibitory postsynaptic currents (IPSCs) recorded from presumed non-GABAergic neurons.

METHODS

Animals

The experiments were performed on glutamate decarboxylase (GAD) 67 green fluorescent protein (GFP) (Δneo) mice, which express GFP under the control of the endogenous GAD67 gene promoter (Tamamaki et al. 2003). In this study, these transgenic mice were referred to as GAD67-GFP knock-in mice. Male heterozygous mice were crossed with Institute of Cancer Research (ICR) wild-type mice to obtain the experimental mice. All animal experiments were approved by the institutional animal care and use committees at Dokkyo Medical University. Care and use of the animals were in accordance with the National Institutes of Health guidelines on animal care and with the guidelines of the International Association for the Study of Pain (Zimmermann 1983).

Preparation of spinal cord slices

The 6- to 8-wk-old animals were intraperitoneally anesthetized with pentobarbital (50 mg/kg) and segments at the lumbar sacral (L₄–S₁) level of the spinal cord were removed. A microslicer (Dosaka EM, Osaka, Japan) was used to cut transverse slices in ice-cold modified Krebs solution [equilibrated with 95% O₂-5% CO₂, containing (in mM) 212 sucrose, 3 KCl, 25 NaHCO₃, 1 NaH₂PO₄, 2 CaCl₂, 1 MgSO₄, and 11 n-glucose; pH 7.4]. The thickness of the slices was 350–450 μm for the electrophysiological experiments and 500 μm for the IHC experiments.

Whole cell recordings

For the electrophysiological experiments, we used a fixed-stage upright microscope (BX50WI; Olympus, Tokyo) equipped with a confocal laser scanning system (FluoView 500; Olympus), infrared differential interference contrast (IR-DIC) optics, and a CCD video camera (IR-CCD 2400; Hamamatsu Photonics, Hamamatsu, Japan). After incubation for 1 h in modified Krebs solution at 37°C, the spinal slices were mounted into the recording chamber on the microscope stage and continuously perfused with Krebs solution [equilibrated with 95% O₂-5% CO₂, containing (in mM) 113 NaCl, 3 KCl, 25 NaHCO₃, 1 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, and 11 n-glucose; pH 7.4].

After the identification of GFP-positive neurons in the SDH by using the confocal laser scanning system as previously described (Fukushima et al. 2005), conventional tight-seal whole cell recordings were obtained from the neurons under IR-DIC optics.

Patch pipettes were fabricated from thin-walled borosilicate glass capillaries pulled on a micropipette puller (P-97; Sutter Instruments, Novato, CA). The pipettes were filled with a solution containing (in mM) 123 K gluconate, 14 KCl, 2 Na gluconate, 1 EGTA (ethylene glycol bis-[β-aminoethyl ether]-N,N,N’,N’-tetraacetic acid), and 10 HEPES [4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid] to record 2-Me-5-HT–induced inward currents. For the IPSC measurements, the pipettes were filled with a solution containing (in mM) 137 KCl, 2 Na gluconate, 1 EGTA, and 10 HEPES. The pH of the internal solution was neutralized to 7.4 with KOH. The direct-current (DC) resistance of the pipettes filled with the internal solution was 5–10 MΩ. The membrane potential value was corrected for junction potential. Fast and slow capacitances were neutralized. The series resistance was compensated by 60% and the access resistance (11–20 MΩ) was continuously monitored; data were discarded when the value changed by >10%.

Glycine receptor blocker strychnine (STR, 0.5–1.0 μM; Sigma, St. Louis, MO), GABAₐ receptor blocker BIC (10 μM; Biomol International, Plymouth Meeting, PA), non-NMDA glutamate receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 μM; Tocris Cookson, Ellisville, MO), voltage-dependent Na⁺-channel blocker tetrodotoxin (TTX, 0.3 μM; Sankyo, Tokyo), 5-HT₃ receptor-selective agonists 2-Me-5-HT (100 μM; Tocris), m-chlorophenylbiguanide hydrochloride (mCPBG, 30 μM; Tocris), 5-HT₃ receptor-selective antagonists ondansetron (ONDAN, 10 μM; Sigma), and tropalyn-1,3,5-dimethylenzolate (TDMB, 10 μM; Tocris) were dissolved in Krebs solution. All drugs except 2-Me-5-HT and mCPBG were applied to the recording chamber by switching the perfusion lines; 2-Me-5-HT and mCPBG were applied locally from another pipette placed near the recorded neuron.

In some experiments, IPSCs were evoked by using a stimulating electrode filled with 1 M NaCl with its tip (∼3 μm in diameter) placed at the dorsolateral margin of the spinal cord, 50–150 μm away from the recording site. Stimulation was carried out at suprathreshold intensity with a 100-μs pulse applied every 10 s. The IPSCs followed high-frequency stimulation (50 Hz) with reduced amplitudes and a constant delay, suggesting that they were evoked monosynaptically.

All recordings were made in the presence of STR and CNQX. Recordings of electrically evoked IPSCs were made in the absence of TTX. For recordings of 5-HT₃ receptor agonist-induced currents and miniature IPSCs (mIPSCs), TTX was added to the perfusate. To confirm that the recorded IPSCs were GABAergic, BIC was added to the perfusate at the end of every experiment.

Currents were recorded at the holding potential of ∼70 mV by using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA). Data were sampled at a rate of 10.0 kHz through a Digidata 1200 interface (Axon Instruments); pCLAMP 9.0.2 (Axon Instruments) and Mini Analysis 6.0.3 (Synaptosoft, Leonia, NJ) were used to analyze the mIPSCs. The threshold for detecting the mIPSCs was set at 3SD of the background noise level (typically 5–10 pA).

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Each event was visually inspected and the events with a rise time <5 ms were accepted as mIPSCs.

**IHC study**

The 500-μm-thick transverse slices were stored in modified Krebs solution containing colchicine (10 μg/mL; Sigma) for 6 h at room temperature. Colchicine, an axonal transport blocking agent, was used to enhance the signal-to-background ratio and clearly show colocalization of endogenous GABA and GFP in the SDH neurons. The slices were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 1% glutaraldehyde for 1 h at room temperature, placed in 20% sucrose in PBS overnight, and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechnical, Tokyo). Cryosections (10-μm thickness) were blocked in 10% normal goat serum in PBS for 1 h at room temperature and then incubated with anti-GABA antibody (A0310, 1:200; Sigma) and anti-GFP antibody (598, 1:500; MBL, Nagoya, Japan) overnight at 4°C. Subsequent detections were performed using Alexa Fluor 488–conjugated goat anti-rabbit IgG (1:200; Invitrogen, Carlsbad, CA) for anti-GFP antibody and Alexa Fluor 594–conjugated goat anti-mouse IgG (1:200; Invitrogen) for anti-GABA antibody. Following incubation in the secondary antibody for 1 h at room temperature, the sections were washed in PBS and coverslipped with Gel Mount (Biomedia, Foster City, CA). The observation was made by using a confocal laser scanning microscope (FluoView FV500; Olympus). The numbers of neurons immunolabeled with both anti-GFP and anti-GABA, with anti-GFP alone, and with anti-GABA alone were counted.

**Single-cell RT-PCR**

After the whole cell recordings, the neurons were aspirated into another pipette following a previously described protocol (Tsuchiya et al. 1999). The collecting pipette had a tip diameter of about 3–5 μm and contained 2 μL of Ca^2+ and Mg^2+ free PBS. The neurons were then injected into thin-walled autoclaved polymerase chain reaction (PCR) tubes under gentle positive pressure and immediately frozen and stored at −80°C until use. The PCR tubes contained 2 μL MgCl_2 (25 mM), 2 μL 10× PCR buffer, 0.5 μL RNase inhibitor (40,000 units/mL), 2 μL nonionic detergent IGEPAL CA-630 (5%), and 5 μL diethylpyrocarbonate (DEPC)-treated water.

On the following day, lysis was performed using IGEPAL CA-630 at room temperature for 5 min; the reverse-transcription (RT) mixture, containing 1 μL oligo (dT) primers (0.5 μg/μL), 2 μL mixed deoxynucleotide triphosphates (dNTPs, 10 mM), 2 μL diithiothreitol (DTT, 0.1 M), 0.5 μL RNase inhibitor (40,000 units/mL), and 1 μL SuperScript II RT (20 units/μL), was then added. The reaction mixture was incubated at 42°C for 50 min and subsequently heat-inactivated at 70°C for 15 min. The total volume of 20 μL complementary DNA (cDNA) was stored at −20°C.

PCR amplifications were performed in a 50-μL solution containing 20 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl_2, 0.2 mM dNTPs, and 2.5 units of Taq DNA polymerase. The concentration of the primers was 20 nM in the first PCR and 200 nM in the second PCR. The primers targeted five genes: neuron-specific enolase (NSE), glutamate decarboxylase 65 (GAD65), glutamate decarboxylase 67 (GAD67), 5-HT_3 receptor (5HT3R), and green fluorescent protein (GFP). The NSE gene was used as a positive control. The primer sequences and product length are listed in Table 1.

The amount of cDNA used for the first PCR varied from 3 to 7 μL and 1 μL of the first PCR product was used for the second PCR. A thermal cycler (GeneAmp 2400; Perkin Elmer) was programmed for 35–40 cycles of 1-min denaturation (94°C), 1-min annealing (54–59°C), and 1-min elongation (72°C). The second PCR products were visualized by electrophoresis on 2% agarose gel with ethidium bromide staining. All products were sequenced with dye terminator chemistry (Applied Biosystems, Foster City, CA) and a DNA sequence modeler (Model 377; Applied Biosystems), and matched the published sequences.

All reagents for the RT-PCR procedure, except the RNase inhibitor (Toyobo, Osaka, Japan) and IGEPAL CA-630 (Sigma), were obtained from Gibco/Invitrogen (Carlsbad, CA).

**Statistical analysis**

Data are presented as the means ± SE (unless otherwise stated). The effects of 2-Me-5-HT and ONDAN on the amplitude of the evoked IPSCs were analyzed by using two-way ANOVA and statistical significance was further evaluated using Tukey’s test for post hoc comparison. Comparison of the effects of 2-Me-5-HT on the mIPSCs was performed using a paired t-test. The Kolmogorov–Smirnov test was used to compare the effect 2-Me-5-HT on the distributions of interevent intervals and amplitude of the mIPSCs. The χ^2 test and Student’s t-test were also used when appropriate. Differences for which P was <0.05 were considered to be significant.

**RESULTS**

**GFP expression and endogenous GABA expression**

Figure 1A(1) is an overview of the distribution of GFP-positive cells in the lumbar spinal cord, showing a dense distribution of GFP-positive cells in the SDH and scattered GFP-positive cells from the deep dorsal horn to the central canal. The observed distribution of the GFP-positive cells is reminiscent of a previous report that many interneuronal so-

**TABLE 1. Primers used for single-cell reverse transcription–polymerase chain reaction (RT-PCR)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Primer Sequence</th>
<th>Sequence Start</th>
<th>Product Length, bp</th>
<th>GenBank Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NSE</em></td>
<td><em>NSE</em>-F</td>
<td>5'-ATAGTGGGCGATGACCTGAC-3'</td>
<td>1031</td>
<td>200</td>
<td>NM_013509</td>
</tr>
<tr>
<td></td>
<td><em>NSE</em>-R</td>
<td>5'-ATGACCTGTCCTGCGTTCC-3'</td>
<td>1230</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>GAD65</em></td>
<td><em>GAD65</em>-F</td>
<td>5'-GAAGAGAACAGGCGCTTGC-3'</td>
<td>3899</td>
<td>181</td>
<td>NM_008078</td>
</tr>
<tr>
<td></td>
<td><em>GAD65</em>-R</td>
<td>5'-TGCTATTAGGAGGTCAGACG-3'</td>
<td>4079</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>GAD67</em></td>
<td><em>GAD67</em>-F</td>
<td>5'-GAGCCTGCGCTGACGTG-3'</td>
<td>1616</td>
<td>231</td>
<td>NM_008077</td>
</tr>
<tr>
<td></td>
<td><em>GAD67</em>-R</td>
<td>5'-GCCACCGCTTGTAGTTTCCC-3'</td>
<td>1846</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>5HT3R</em></td>
<td><em>5HT3</em>-F</td>
<td>5'-CAGTAGTCGGTGAGCAGGATC-3'</td>
<td>1168</td>
<td>223</td>
<td>NM_013561</td>
</tr>
<tr>
<td></td>
<td><em>5HT3</em>-R</td>
<td>5'-GCTTGGGAGAAGGCGTTATC-3'</td>
<td>1390</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*NSE*, neuron-specific enolase gene; *GAD65*, glutamate decarboxylase 65 gene; *GAD67*, glutamate decarboxylase 67 gene; *5HT3R*, 5-hydroxytryptamine (5-HT, serotonin) type 3 receptor gene. Two 5-HT_3 receptor subunits were cloned: subunit A (5-HT_3A) and subunit B (5-HT_3B). Although expression of 5-HT_3A subunits alone yields functional 5-HT_3 receptors, it has been suggested that 5-HT_3A subunits modify the physiological and pharmacological properties of 5-HT_3 receptors (Davies et al. 1999; Hapfelmeier et al. 2003). In this study, we attempted to detect the 5-HT_3A subunits. The primers were used for both the first and second PCR amplifications. Each primer was individually used in the second PCR. The concentration of primers was 20 nM each in the first PCR and 200 nM in the second PCR.
mata in the substantia gelatinosa show GABA immunoreactivity (Todd and McKenzie 1989; Todd and Spike 1993).

To assess the correlation of GFP fluorescence with endogenous GABA expression in the SDH, we performed double-immunostaining with anti-GFP and anti-GABA antibodies. Figure 1, A(2)–A(4) shows representative double-staining IHC images for GFP and GABA. In 16 sections randomly selected from three mice, we approximately estimated that of the 512 lamina II neurons immunostained for GFP, 498 (97.2%) were also immunolabeled for GABA. Conversely, of the 588 lamina II neurons immunostained for GABA, 493 (84.4%) were also immunolabeled for GFP.

**GFP expression and RT-PCR analysis of GAD65 and GAD67 mRNAs**

In the present study, we used GAD67-GFP knock-in mice expressing GFP specifically in GABAergic neurons because
GFP is expressed under the control of the endogenous GAD67 gene promoter. In addition to GAD67, however, previous studies have found another isoform of the GABA-synthesizing enzyme, GAD65 (Bu et al. 1992). In the dorsal horn, some GABAergic neurons contain high levels of either GAD65 or GAD67, whereas others contain both isoforms in equal proportion (Feldblum et al. 1995; Mackie et al. 2003). Additionally, these two isoforms are involved in different physiological functions and their expression in the SDH is differentially regulated (Mackie et al. 2003; Moore et al. 2002). This prompted us to evaluate the correlation of GFP expression with the expression pattern of GAD65 and GAD67 mRNAs by means of single-cell RT-PCR.

We performed RT-PCR on the total RNA isolated from individual GFP-positive neurons sampled from the SDH. After amplification by a second cycle of PCR, both GAD65 and GAD67 sequence-specific PCR products were observed in 36 of the 48 GFP-positive neurons, as shown in Fig. 1B (cell 1). In 9 of the 48 GFP-positive neurons, GAD67 alone was detected (Fig. 1B, cell 2). In the remaining 3 GFP-positive neurons, PCR products of neither GAD65 nor GAD67 were detected (Fig. 1B, cell 3).

For comparison, samples from GFP-negative neurons were amplified using identical experimental protocols. In 30 of the 45 GFP-negative neurons, neither GAD65 nor GAD67 was detected. In the remaining 15 GFP-negative neurons, 9 showed GAD65 products and 6 showed GAD67 products.

**5-HT3 receptor-mediated inward currents in GABAergic neurons**

Tight-seal whole cell recordings were made from GFP-positive neurons. Local application of 2-Me-5-HT was carried out by using a glass pipette (20- to 50-μm orifice) placed near the recorded neurons in the presence of STR, CNQX, and TTX. At a concentration of 100 μM, 2-Me-5-HT elicited inward currents in a considerable number of neurons. A representative recording of a 2-Me-5-HT–induced inward current in a GFP-positive neuron is shown in Fig. 2A. Single-cell RT-PCR analysis indicated that the GFP-positive neuron exhibited PCR products of GAD67 and 5HT3R (Fig. 2B).

As shown in Table 2, the PCR product of 5HT3R was detected in all the tested GFP-positive and GFP-negative neurons exhibiting inward currents in response to 2-Me-5-HT. Table 2 also shows the incidence and amplitude of 2-Me-5-HT–induced inward currents as well as the passive membrane properties for GFP-positive and GFP-negative neurons. The incidence of inward currents was statistically equivalent for the GFP-positive and GFP-negative neurons (P = 0.288). The mean amplitude of the 2-Me-5-HT–mediated inward currents was smaller in the GFP-positive neurons than in the GFP-negative neurons. The time constant of a single exponential fitted to the decay phase of the 2-Me-5-HT–induced currents was not significantly different between the groups. Membrane capacitance of the GFP-positive neurons was significantly larger than that of the GFP-negative neurons. Membrane resistance was not significantly different between the groups. Inward currents induced by local application of 2-Me-5-HT and the results of single-cell RT-PCR indicate that 5-HT3 receptors are expressed in somadendritic regions of the neurons in the SDH.

**Effects of 2-Me-5-HT on electrically evoked GABAergic IPSCs**

In the next stage of experiments, we analyzed the effects of 2-Me-5-HT on GABAergic synaptic transmission in the SDH. GABAergic autaptic connections have been described in several regions of the CNS (Bekkers and Stevens 1991; Bergevin et al. 2002; Michel and Trudeau 2000). To reduce the possibility of recording IPSCs mediated by GABAergic autapses, we recorded IPSCs from GFP-negative neurons in the SDH. Most of the GFP-negative neurons can be presumed to be non-GABAergic. Although it is not known whether the GFP-negative neurons are excitatory or inhibitory, the recording of GABAergic IPSCs indicates that the actions of 5-HT3 receptors on the synaptic transmission between GABAergic neurons and neurons of other phenotypes.

Tight-seal whole cell recordings were made from the GFP-negative presumably non-GABAergic neurons located in the SDH. In the presence of CNQX, to block glutamatergic excitatory synapses, and STR, to block glycineric inhibitory synapses, postsynaptic currents were evoked by electrical stimulation of internuncial neurons in the spinal slices. These postsynaptic currents were abolished by BIC (10 μM; data not shown) and were thus identified as GABAergic IPSCs.

The graph in Fig. 3A shows the time course of the effects of 2-Me-5-HT on the amplitude of the evoked GABAergic IPSCs. The amplitude of the IPSCs was 139.1 ± 3.6 pA before 2-Me-5-HT application (n = 12) and increased to 242 ± 36 pA (177 ± 9% of the control, P < 0.01) 10 s after the application. The amplitude then decreased rapidly, suggesting that desensitization may begin during the application of an agonist. The 5-HT3 recep-
Effects of 2-Me-5-HT on the frequency and amplitude of GABAergic mIPSCs

Spontaneously occurring mIPSCs were recorded from the GFP-negative neurons in the presence of TTX, CNQX, and STR. These mIPSCs were completely abolished by BIC (data not shown) and were thus identified to be GABA<sub>A</sub>-mediated mIPSCs.

Figure 4, A–D exemplifies the effects of 2-Me-5-HT on the GABAergic mIPSCs. The frequency of the mIPSCs increased immediately after 2-Me-5-HT application and gradually decreased during the application (Fig. 4A). The amplitude of the mIPSCs did not show any apparent changes in response to 2-Me-5-HT (Fig. 4B). Cumulative histograms of interevent intervals of the mIPSCs indicate a significant increase in the mIPSC frequency (P < 0.05, Fig. 4C). On the other hand, cumulative histograms of the mIPSC amplitudes indicate that the amplitudes did not change significantly (P = 0.42, Fig. 4D). The same results as depicted in Fig. 4, A–D were obtained for seven neurons. The application of 2-Me-5-HT significantly increased the frequency of the mIPSCs to 183 ± 29.3% of the control (Fig. 4E, n = 7, P < 0.05), although their mean amplitudes were not affected (Fig. 4F, n = 7, P = 0.48).

In the presence of ONDAN, the application of 2-Me-5-HT did not exert facilitatory actions on the frequency of the mIPSCs, as exemplified by Fig. 5, A–D. The same observations were made in six neurons. In the presence of ONDAN, 2-Me-5-HT did not affect the mIPSC frequency (Fig. 5E, P = 0.48) or the amplitude (Fig. 5F, P = 0.93).

![Fig. 3. Effects of 2-Me-5-HT on the amplitude of GABAergic inhibitory postsynaptic currents (IPSCs) evoked by electrical stimulation.](http://jn.physiology.org/)

Table 2. 2-Me-5-HT–induced inward currents and passive membrane properties of GFP-positive and GFP-negative neurons in the SDH

<table>
<thead>
<tr>
<th>Property</th>
<th>GFP-Positive Neuron</th>
<th>GFP-Negative Neuron</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occurrence of neurons with 2-Me-5-HT–induced currents</td>
<td>34.0% (18/53)</td>
<td>43.9% (25/57)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Occurrence of 5HT3R mRNA</td>
<td>100% (16/16)</td>
<td>100% (12/12)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Occurrence of GAD67 mRNA</td>
<td>93.8% (15/16)</td>
<td>16.7% (2/12)</td>
<td>*P &lt; 0.05</td>
</tr>
<tr>
<td>Amplitude of 2-Me-5-HT–induced currents, pA/pF</td>
<td>1.9 ± 0.1 (18)</td>
<td>2.6 ± 0.5 (25)</td>
<td>*P &lt; 0.05</td>
</tr>
<tr>
<td>Decay time constant of 2-Me-5-HT–induced currents, ms</td>
<td>504.0 ± 152.0 (18)</td>
<td>466.0 ± 80.0 (25)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Membrane capacitance, pF</td>
<td>46.6 ± 3.7 (18)</td>
<td>34.2 ± 1.8 (25)</td>
<td>*P &lt; 0.05</td>
</tr>
<tr>
<td>Membrane resistance, MΩ</td>
<td>439.7 ± 73.9 (18)</td>
<td>603.6 ± 69.9 (25)</td>
<td>P &gt; 0.05</td>
</tr>
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</table>


gA values are either percentages or means ± SE; number of observations is indicated in parentheses. Single-cell RT-PCR analysis for 5HT3R and GAD67 in GFP-positive and GFP-negative neurons that exhibited inward currents in response to 2-Me-5-HT is also shown. *Considered to be significant in this study.
These observations strongly suggest that 5-HT3 receptors are the presence of TDMB (10 μMIPSCs was not affected by mCPBG (Fig. 6C, D). In both cases, 2-Me-5-HT (100 μM) was applied for 100 s, as indicated by the horizontal bar. The dots on graph A indicate the frequency of the mIPSCs increased rapidly in response to application of 2-Me-5-HT and decreased gradually even in the presence of the agonist (A). The amplitude of the mIPSCs did not show any appreciable changes in response to 2-Me-5-HT (B). In both cases, 2-Me-5-HT (100 μM) was applied for 100 s, as indicated by the horizontal bar. The dots on graph A indicate the frequency of the mIPSCs measured every 10 s. The dots and vertical bars on graph B indicate the mean and SE of the mIPSC amplitude during a 10-s period. The tracings in the inset are sample recordings spanning 20-s periods indicated by brackets before (1) and during (2) application of 2-Me-5-HT. The histograms labeled "control" were constructed from 51 events collected during a 90-s period immediately before the start of 2-Me-5-HT application. The histograms labeled "2-Me-5-HT" were constructed from 81 events collected during a 90-s period immediately after the start of 2-Me-5-HT application. The histograms labeled "2-Me-5-HT" and "control" were constructed from 51 events collected during a 90-s period immediately before and after the start of 2-Me-5-HT application. The bars and vertical lines indicate the means ± SE. 2-Me-5-HT increased the frequency of the mIPSCs (E) without affecting their amplitude (F). *P < 0.05, significant; NS, not significant.

Additionally, we examined the actions of the other agonist (mCPBG) and antagonist (TDMB), specific for 5-HT3 receptors. The application of mCPBG (30 μM) increased the mIPSC frequency from 1.09 ± 0.06 to 2.38 ± 0.39 Hz (246 ± 42% of the control, Fig. 6B, n = 6, P < 0.05). The amplitude of the mIPSCs was not affected by mCPBG (Fig. 6C, P = 0.704). In the presence of TDMB (10 μM), the application of mCPBG did not exert any apparent effects on the mIPSC frequency (Fig. 6E, n = 7, P = 0.596) or amplitude (Fig. 6F, P = 0.796). These observations strongly suggest that 5-HT3 receptors are expressed in the presynaptic terminals of GABAergic neurons and that 5-HT3 receptors exert facilitatory actions on GABA release in the SDH.

**DISCUSSION**

In this study, we found that GFP-positive GABAergic and GFP-negative presumably non-GABAergic neurons in the SDH express 5-HT3 receptors in their somadendritic regions and respond with a transient inward current to local application of the 5-HT3 receptor-selective agonist 2-Me-5-HT. In addition, the amplitude of GABA_A-receptor-mediated IPSCs evoked by electrical stimulation of internuncial neurons, which were recorded from GFP-negative presumably non-GABAergic neurons, was increased by 2-Me-5-HT. Furthermore, the frequency but not the amplitude of the mIPSCs was increased by 2-Me-5-HT, suggesting that 5-HT3 receptors located on the presynaptic terminals augment the release of GABA in the SDH.

**Correlation of GFP fluorescence with expressions of GAD65 and GAD67 mRNAs and endogenous GABA**

GABA is synthesized from glutamate by the enzyme GAD. Two isoforms of GAD have been identified based on their molecular weights: GAD65 and GAD67 (Bu et al. 1992).
single-cell RT-PCR analysis indicates that one fifth of the GFP-positive neurons expressed GAD67 mRNA alone, and three fourths expressed both GAD67 and GAD65 mRNAs. In the remaining GFP-positive neurons, neither GAD67 mRNA nor GAD65 mRNA could be detected. As for the GFP-negative neurons, two thirds did not express either GAD67 mRNA or GAD65 mRNA, whereas the remainder expressed mRNA of either isoform.

These observations, although limited, seem to be in accordance with previous investigations of the distribution of GAD65 and GAD67 in the spinal dorsal horn (Feldblum et al. 1995; Mackie et al. 2003). Allegedly, almost all GABAergic neurons in the CNS express both forms of GAD; however, the relative expression level of the isoforms is significantly different from area to area as well as from neuron to neuron in the CNS (Soghomonian and Martin 1998). Additionally, differential regulation of the expressions of GAD65 and GAD67 in the SDH has been suggested (Mackie et al. 2003; Moore et al. 2002). A more recent double-labeling IHC analysis performed on the same strain of GAD67-GFP knock-in mice as in our experiments showed that >91.2% of the neurons immunostaining for GFP are also immunoreactive for GAD67 (Huang et al. 2008).

Some GFP-positive neurons did not express GAD67 mRNA. This might be accounted for as “false-negative” because of the insufficient number of mRNA copies in single cells, a limitation generally associated with single-cell RT-PCR. It should
also be noted that the PCR products of GAD67 mRNA were detected in some GFP-negative neurons; this might be related to the possibility that transcription of GAD67 mRNA and translation of GAD67 mRNA into protein are differently regulated (Rimvall et al. 1993). Although such drawbacks cannot be eliminated, our single-cell RT-PCR analysis, taken together with the previous IHC analysis (Huang et al. 2008), indicates that GFP expression is under the proper control of GAD67 promoters.

**Functional 5-HT3 receptors are expressed in axon terminals and somadendritic regions of GABAergic neurons in the SDH**

Local application of 2-Me-5-HT increased the frequency of GABA_A receptor-mediated mIPSCs without affecting their amplitudes. These facilitatory actions of 2-Me-5-HT were blocked by ONDAN. These results strongly suggest that activation of presynaptically located 5-HT_3 receptors increases the release of GABA in the SDH.

In their IHC investigation of the SDH, Maxwell et al. (2003) found that axons immunolabeling for both 5-HT_3 receptor and GAD67 are few in number, although they exist. In contrast, we observed that GABAergic neurons in the SDH expressed presynaptic 5-HT_3 receptors. The reasons for this discrepancy are presently not clear. One reason could be the different methods used (IHC staining vs. whole cell recording combined with single-cell RT-PCR analysis). Another possibility is that the frequency of the GABAergic mIPSCs recorded from non-GABAergic neurons was increased by the 5-HT_3 receptor agonists, suggesting that GABAergic terminals targeting non-
GABAergic neurons express 5-HT₃ receptors. The mechanisms for target-specific regulation of receptor expression in the presynaptic terminals might explain the discrepancy (Reyes et al. 1998; Shigemoto et al. 1996). More recently, Abe et al. (2009) reported that a 5-HT₃ receptor-specific agonist induced inward currents in neurons with particular morphological characteristics (such as islet cells). Because a previous IHC study has shown that many of the islet cells are GABA-immunoreactive (Todd and McKenzie 1989), it is speculated that 5-HT₃ receptors are expressed on GABAergic neurons. Our present results provide direct evidence of the presynaptic and somadendritic expression of 5-HT₃ receptors on GABAergic neurons in the SDH.

More recently, Huang et al. (2008) applied single-cell RT-PCR analysis to dissociated neurons and showed that 28.1% of the GABAergic neurons in the SDH express 5-HT₃ receptor mRNA. Our single-cell RT-PCR analysis of in situ GABAergic neurons located in the spinal slices is comparable with their report. Additionally, our whole cell recordings revealed that 2-Me-5-HT elicited a transient inward current in a considerable number of GFP-positive neurons. Evidently, functional 5-HT₃ receptors are present in both presynaptic terminals and cell bodies of a subpopulation of GABAergic neurons in the SDH.

5-HT₃ receptors presynaptically facilitate GABAergic inhibitory synaptic transmission in the SDH

We observed that 2-Me-5-HT increased the frequency of the GABAergic mIPSCs without affecting their amplitude. Similar to our observation of the presynaptic actions of 5-HT₃ receptors, previous studies have reported these actions in other regions of the CNS such as the hippocampus and amygdala (Dorostkar and Boehm 2007; Ferezou et al. 2002; Koyama et al. 2000; Turner et al. 2004; Zhou and Hablitz 1999). In addition, Dorostkar and Boehm (2007) showed that in hippocampal GABAergic autapses, activation of 5-HT₃ receptors suppresses action potential (AP)–evoked IPSCs and increases the frequency of mIPSCs. These authors suggested that presynaptic 5-HT₃ receptors control the spontaneous and AP-dependent release of GABA in the hippocampus differently. In the SDH, however, we observed that 2-Me-5-HT increased the amplitude of the GABA_A receptor-mediated IPSCs evoked by electrical stimulation of interneuronal neurons in the spinal slices. This might be explained by the activation of presynaptic 5-HT₃ receptors, resulting in enhanced release probability of GABA. Another possibility is that the activation of somadendritic 5-HT₃ receptors depolarizes GABAergic neurons, resulting in the activation of an increased number of neurons by an electrical stimulating pulse.

With regard to the effects of 5-HT on neuronal excitability, it has been suggested that the activation of 5-HT₃ receptors excites GABAergic neurons in several brain regions such as the hippocampus and cerebral cortex (Alreja 1996; Ferezou et al. 2002; Kawa 1994; Puig et al. 2004). Excitatory actions of 5-HT on SDH neurons have also been described, although the responsible receptor subtypes have not been clarified (Hori et al. 1996; Todd and Millar 1983,1984).

Functional significance

The behavioral investigations and electrophysiological experiments have shown that analgesia induced by the activation of 5-HT₃ receptors is blocked by spinal application of GABA_A receptor antagonists (Alhaieder et al. 1991; Cui et al. 1999; Giordano 1991; Giordano and Schultea 2004; Lin et al. 1994; Millan 2002; Peng et al. 2001; Yang et al. 1998). One possible mechanism is that 5-HT excites GABAergic neurons in the SDH, resulting in an increased release of GABA and in turn exhibiting postsynaptic and/or presynaptic modulatory actions on nociceptive transmission (Alhaieder et al. 1991; Lin et al. 1994, 1996; McGowan and Hammond 1993; Tanimoto et al. 2004). Supporting this hypothesis, a previous analysis by using microdialysis has shown that activation of 5-HT₃ receptors increases the concentration of GABA in the spinal dorsal horn (Kawamata et al. 2003). However, there has been no synaptic evidence for the involvement of GABA in 5-HT receptor-
mediated antinociception at the spinal cord level. Our present findings might provide a synaptic basis underlying the antino-
ceptive action of spinal 5-HT_3_ receptors.

Application of 2-Me-5-HT induced inward currents not only in GFP-positive neurons but also in GFP-negative neurons. Furthermore, the amplitude of the 2-Me-5-HT-induced inward currents was larger in the GFP-negative neurons than that in GFP-positive neurons, although the incidence of inward cur-
rents was not significantly different between the groups. At present, we do not know whether these GFP-negative neurons are excitatory or inhibitory; therefore the physiological func-
tions of 5-HT_3_ receptors on GFP-negative neurons in the SDH are not clear. Recently, it was suggested that many of the 5-HT_3_ receptor-expressing axon terminals in the spinal dorsal horn are excitatory and originate from interneurons in the dorsal horn (Conte et al. 2005; Maxwell et al. 2003). Addi-
tionally, we had previously shown that 2-Me-5-HT increases the frequency but not the amplitude of glutamatergic miniature excitatory synaptic currents recorded from spinal dorsal horn neurons (Hori and Endo 1992). Therefore we speculate that a considerable number of the GFP-negative neurons expressing 5-HT_3_ receptors, as observed in the experiments, are excitatory neurons. Controversial reports have shown that activation of 5-HT_3_ receptors in the spinal cord exerts pronociceptive ac-
tions (Danzebrink and Gebhart 1991; Miranda et al. 2006; Oatway et al. 2004; Oyama et al. 1996; Sufka et al. 1992; Zeitz et al. 2003). For example, Zeitz et al. (2003) showed that the persistent phase but not the acute phase of formalin-evoked responses is reduced in 5-HT_3_ receptor knockout mice. Oatway et al. (2004) reported that mechanical allodynia induced by tissue injury is attenuated by intrathecal administration of ONDA\N. These studies suggest that 5-HT_3_ receptors are in-
volved in tissue injury–induced persistent pain. The GFP-

negative neurons expressing 5-HT_3_ receptors found in our experiments may be functionally involved in such pronociceptive ac-
tions of 5-HT.

Origin of GABAergic synaptic terminals expressing 5-HT_3_ receptors

We recorded GABA_A receptor-mediated IPSCs from SDH

neurons. The origins of presynaptic terminals responsible for these GABAergic IPSCs are not clear. It has been recently reported that GABAergic neurons in the supraspinal structures send descending fibers to the spinal cord and make monosyn-
aptic inhibitory connections with neurons in the SDH (Antal et al. 1996; Kato et al. 2006). On the other hand, GABAergic neurons are known to be densely located in the SDH (Mackie et al. 2003; Makinae et al. 2000; Mitchell et al. 1993; Todd and McKenzie 1989; Todd and Spike 1993) and, in fact, we observed a high concentration of GFP-positive GABAergic somata in the SDH.

Conclusion

We have found that a subpopulation of GFP-positive neu-
rons in the SDH express functional 5-HT_3_ receptors both in somadendritic regions and at presynaptic terminals. Addition-
ally, we had previously shown that a significant proportion of

enkephalinergic neurons in the SDH express 5-HT_3_ receptors (Tsuchiya et al. 1999). Enkephalin is also localized in high concentrations in the SDH and is involved in the modulation of nociceptive transmission (Du and Dubois 1988; Hokfelt et al. 1977; Tsuchiya et al. 1999). Furthermore, IHC investigations have shown that many SDH neurons contain both enkephalin and GABA, some of the SDH neurons contain enkephalin but not GABA, and others contain GABA but not enkephalin (Huang et al. 2008; Jo et al. 1998; Todd et al. 1992). Thus it is speculated that at least some of the GFP-negative neurons in the SDH are enkephalin-containing inhibitory interneurons and that GABAergic and/or enkephalineric SDH neurons expressing 5-HT_3_ receptors may provide a synaptic basis for the mechanism underlying the antinociceptive action of 5-HT.

Finally, as illustrated in Fig. 7, our present observations indicate that a subpopulation of GABAergic interneurons in the superficial dorsal horn express 5-HT\_3\_ receptors in the soma-
dendritic and presynaptic regions. Somadendritic 5-HT_3_ receptors regulate the excitability of GABAergic interneurons and presynaptic 5-HT_3_ receptors regulate the exocytotic process of GABA release. GABAergic interneurons expressing 5-HT_3_ receptors underlie the mechanisms for 5-HT spinal antinoci-
ception. Spinal nociceptive transmission occurs under the modula-
tion by 5-HT through various subtypes of 5-HT receptors, including 5-HT_3_ receptors, that indirectly inhibit nociceptive transmission by activation of inhibitory interneurons.

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