Facilitatory actions of serotonin type-3 receptors on GABAergic inhibitory synaptic transmission in the spinal superficial dorsal horn

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Running head: 5-HT3 receptors on GABAergic neurons in the SDH

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ABSTRACT

Analgesic effects of serotonin (5-hydroxytryptamine, 5-HT) type 3 (5-HT3) receptors may involve the release of γ-amino-butyric acid (GABA) in the spinal dorsal horn. However, the precise synaptic mechanisms for 5-HT3 receptor-mediated spinal analgesia are not clear. In this study, we investigated whether GABAergic neurons in the superficial dorsal horn (SDH) express functional 5-HT3 receptors and how these 5-HT3 receptors affect GABAergic inhibitory synaptic transmission in the SDH, by using slice preparations from adult glutamate decarboxylase 67-green fluorescent protein (GAD67-GFP) knock-in mice. Tight-seal whole-cell recordings from GFP-positive and negative neurons showed that 5-HT3 receptor-specific agonist 2-methyl-serotonin (2-Me-5-HT) induced inward currents in a substantial population of both GFP-positive and negative neurons. Additionally, we confirmed expression of 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 clues 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 central nervous system (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; Tsuchiya 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 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 spinal dorsal horn neurons to noxious cutaneous stimuli and locally applied N-methyl D-aspartate (NMDA) and substance P are inhibited by iontophoretically 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 spinal dorsal horn: 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 spinal dorsal horn 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 antinociceptive 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 antinociception via GABA_A receptors (Alhaider et al. 1991; Kawamata et al. 2003; Lin et al. 1994; Lin et al. 1996; McGowan and Hammond 1993; Tanimoto et al. 2004).

The expression of 5-HT3 receptors on GABAergic neurons has been shown in central brain
regions such as the telencephalon (Morales et al. 1996; Morales and Bloom 1997) 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 GABA\textsubscript{A} 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 GABA\textsubscript{A} receptor-mediated inhibitory postsynaptic currents (IPSCs) recorded from presumed non-GABAergic neurons.
MATERIALS AND METHODS

Animals

The experiments were performed on glutamate decarboxylase (GAD) 67 green-fluorescent protein (GFP) (Δneo) mice, which express GFP under the control of 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 (NIH) guidelines on animal care and with the guidelines of the International Association for the Study of Pain (IASP) (Zimmermann 1983).

Preparation of spinal cord slices

The animals (6- to 8-week-old) were anesthetized with pentobarbital (50 mg/kg) intraperitoneally and segments at the lumbosacral (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 and 5% CO₂; containing 212-mM sucrose, 3-mM KCl, 25-mM NaHCO₃, 1-mM NaH₂PO₄, 2-mM CaCl₂, 1-mM MgSO₄, 11-mM D-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, Japan) 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 and 5% CO₂; containing 113-mM NaCl, 3-mM KCl, 25-mM NaHCO₃, 1-mM NaH₂PO₄, 2-mM CaCl₂, 1-mM MgCl₂, and 11-mM D-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, USA). The pipettes were filled with a solution containing 123-mM K gluconate, 14-mM KCl, 2-mM Na gluconate, 1-mM EGTA (ethylene glycol bis[β-aminoethyl ether]-N,N',N,N'-tetraacetic acid), and 10-mM HEPES (4-[2-hydroxyethyl]-1-piperazinethanesulfonic acid) to record 2-Me-5-HT-induced inward currents. For the IPSC measurements, the pipettes were filled with a solution containing 137-mM KCl, 2-mM Na gluconate, 1-mM EGTA, and 10-mM 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 and data were discarded when the value changed by more than 10%.

Glycine receptor blocker strychnine (STR, 0.5–1.0 µM; Sigma, St. Louis, MO, USA),
GABA<sub>A</sub> receptor blocker BIC (10 µM; Biomol International, Plymouth Meeting, PA, USA), non-NMDA glutamate receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 µM; Tocris Cookson, Inc., Ellisville, MO, USA), voltage-dependent Na<sup>+</sup>-channel blocker tetrodotoxin (TTX, 0.3 µM; Sankyo, Tokyo, Japan), 5-HT<sub>3</sub> receptor-selective agonists 2-Me-5-HT (100 µM; Tocris), m-chlorophenylbiguanide hydrochloride (mCPBG, 30 µM; Tocris), 5-HT<sub>3</sub> receptor-selective antagonists ondansetron (ONDAN, 10 nM; Sigma) and tropanyl-3,5-dimethylbenzoate (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-HT<sub>3</sub> 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, USA). Data were sampled at a rate of 10.0
kHz through a Digidata 1230 interface (Axon Instruments); pCLAMP 9.0.2 (Axon Instruments) and Mini Analysis 6.0.3 (Synaptosoft, Inc., Leonia, NJ, USA) were used to analyze the mIPSCs. The threshold for detecting the mIPSCs was set at trice the standard deviation of the background noise level (typically 5–10 pA). Each event was visually inspected and the events with a rise time shorter than 5 ms were accepted as mIPSCs.

**IHC study**

The 500-µm-thick transverse slices of 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 Finetechincal, Tokyo, Japan). 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, USA) 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 cover-slipped with Gel Mount (Biomedica, Foster City, CA, USA). 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 reverse transcription polymerase chain reaction**

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 Ca²⁺-free and Mg²⁺-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 MgCl₂ (25 mM), 2-µL 10X 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 dithiothreitol (DTT, 0.1 M), 0.5-µL RNase inhibitor (40,000 units/mL), and 1-µL SuperScript II RT (200 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.

PCRs were performed in a 50-µL solution containing 20-mM Tris-HCl, 50-mM KCl, 2.5-mM MgCl₂, 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-HT₃ 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
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, USA) and a DNA sequencer (Model 377; Applied Biosystems), and matched the published sequences.

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

**Statistical analysis**

Data are presented as the mean ± SEM (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 analysis of variance (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 chi-square test and Student’s t-test were also used when appropriate. Differences for which P was less than 0.05 were considered to be significant.
RESULTS

GFP expression and endogenous GABA expression

<<Figure 1>>

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 somata 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. Figures 1A(2)–(4) show representative double-staining IHC images for GFP and GABA. In 16 sections randomly selected from 3 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 \textit{GAD65} and \textit{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 \textit{GAD65} and \textit{GAD67} sequence-specific PCR products were observed in 36 of the 48 GFP-positive neurons, as shown in Figure 1B (cell #1). In 9 of the 48 GFP-positive neurons, \textit{GAD67} alone was detected (Fig. 1B, cell #2). In the remaining 3 GFP-positive neurons, PCR products of neither \textit{GAD65} nor \textit{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 \textit{GAD65} nor \textit{GAD67} was detected. In the remaining 15 GFP-negative neurons, 9 showed \textit{GAD65} products and 6 showed \textit{GAD67} products.

\textbf{5-HT\textsubscript{3} receptor-mediated inward currents in GABAergic neurons}

<<Figure 2>>  <<Table 2>>

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 (20–50 µm orifice) placed near the recorded neurons \textbf{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 Figure 2A. Single-cell RT-PCR analysis indicated that the GFP-positive neuron exhibited PCR products of \textit{GAD67} and \textit{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 somatodendritic regions of the neurons in the SDH.

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

<<Figure 3>>

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 GABA<sub>A</sub> receptor-mediated IPSCs from GFP-negative neurons allows
analysis of the actions of 5-HT\textsubscript{3} 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 glycinergic inhibitory synapses, postsynaptic currents were evoked by electrical stimulation of internuncial neurons in the spinal slices. These postsynaptic currents were abolished by BIC (10 \, \mu M, data not shown) and were thus identified as GABA\textsubscript{A} receptor-mediated IPSCs.

The graph in Figure 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-HT\textsubscript{3} receptor-selective antagonist ONDAN clearly blocked the facilitatory action of 5-Me-5-HT (n = 11, \(P > 0.05\)) at a concentration of 10 nM, as illustrated in Figure 3B.

**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\textsubscript{A} receptor-mediated mIPSCs.

Figures 4A–D exemplify 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 Figures 4A–D were obtained for 7 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, $n = 7$, $P = 0.48$).

<<Figure 5>>

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 Figures 5 A–D. The same observations were made in 6 neurons. In the presence of ONDAN, 2-Met-5-HT did not affect the mIPSC frequency (Fig. 5E, $P = 0.48$) or the amplitude (Fig. 5F, $P = 0.93$).

<<Figure 6>>

Additionally, we examined the actions of other agonist, mCPBG and antagonist, TDMB, specific for 5-HT$_3$ 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-HT$_3$ receptors are expressed in the presynaptic terminals of GABAergic neurons and that 5-HT$_3$ 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-HT$_3$ receptors in their somadendritic regions and respond with a transient inward current to local application of 5-HT$_3$ 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-HT$_3$ 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, namely GAD65 and GAD67 (Bu et al. 1992). Our single-cell RT-PCR analysis indicates that one-fifth of the GFP-positive neurons expressed GAD67 mRNA alone, and three-fourth 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-third did not express GAD67 mRNA or GAD65 mRNA, whereas the reminder 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 more than 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-HT$_3$ 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-HT$_3$ receptors. The mechanisms for target-specific regulation of receptor expression in the presynaptic terminals might explain the discrepancy (Shigemoto et al. 1996; Reyes et al. 1998).

More recently, Abe et al. (2009) have reported that a 5-HT$_3$ receptor-specific agonist induced inward currents in neurons with particular morphological characterization (such as islet cells). As 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-HT$_3$ 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-HT$_3$ 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) have shown that in hippocampal GABAergic autapses, activation of 5-HT₃ receptors suppresses action potential-evoked IPSCs, and increases the frequency of mIPSCs. These authors suggested that presynaptic 5-HT₃ receptors control the spontaneous and action-potential-dependent release of GABA in the hippocampus differently. In the SDH, however, we observed that 2-Me-5-HT increased the amplitude of the GABAₐ receptor-mediated IPSCs evoked by electrical stimulation of internuncial 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 1984; 1983).
**Functional significance**

The behavioral investigations and electrophysiological experiments have shown that analgesia induced by the activation of 5-HT$_3$ receptors is blocked by spinal application of GABA$_A$ receptor antagonists (Alhaider 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 (Alhaider et al. 1991; Lin et al. 1994; Lin et al. 1996; McGowan and Hammond 1993; Tanimoto et al. 2004). Supporting this hypothesis, a previous analysis by using microdialysis has shown that activation of 5-HT$_3$ 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 antinociceptive action of spinal 5-HT$_3$ receptors.

Application of 2-Me-5-HT induced inward currents not only in the GFP-positive neurons but also in the GFP-negative neurons. Furthermore, the amplitude of the 2-Me-5-HT-induced inward currents was larger in the GFP-negative neurons than in GFP-positive neurons, although the incidence of inward currents 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 functions 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 (Maxwell et al. 2003; Conte et al. 2005). Additionally, we had previously shown that 2-Me-5-HT increases the frequency but not the amplitude of glutamatergic miniature excitatory synaptic currents (EPSCs) recorded...
from spinal dorsal horn neurons (Hori and Endo 1992). Therefore, we speculate that a considerable number of the GFP-negative neurons expressing 5-HT₃ receptors, as observed in the experiments, are excitatory neurons. Controversial reports have shown that activation of 5-HT₃ receptors in the spinal cord exerts pronociceptive actions (Danzebrink and Gebhart 1991; Miranda et al. 2006; Oyama et al. 1996; Sufka et al. 1992; Zeitz et al. 2003; Oatway et al. 2004). For example, Zeitz et al. (2003) have shown that the persistent phase but not the acute phase of formalin-evoked responses is reduced in 5-HT₃ receptor knockout mice. Oatway et al. (2004) have reported that mechanical allodynia induced by tissue injury is attenuated by intrathecal administration of ONDAN. These studies suggest that 5-HT₃ receptors are involved in tissue injury-induced persistent pain. The GFP-negative neurons expressing 5-HT₃ receptors found in our experiments may be functionally involved in such pronociceptive actions of 5-HT.

**Origin of GABAergic synaptic terminals expressing 5-HT₃ 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 monosynaptic 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 neurons in the SDH express functional 5-HT$_3$ receptors both in somadendritic regions and at presynaptic terminals. Additionally, 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 (Jo et al. 1998; Todd et al. 1992; Huang et al. 2008). 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 enkephalinergic 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 Figure 7, our present observations indicate that a subpopulation of GABAergic interneurons in the superficial dorsal horn express 5-HT$_3$ receptors in the somadendritic 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 antinociception. Spinal nociceptive transmission occurs under the modulation 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.
ACKNOWLEDGEMENTS

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**Yang S, Guo YQ, Kang YM, Qiao JT, Laufman LE, and Dafny N.** Different GABA-receptor


FIGURE LEGENDS

Figure 1.

Distribution of GFP-positive neurons and double-staining IHC colocalization of GFP expression with endogenous GABA (A) and RT-PCR analysis of the expressions of GAD65 and GAD67 mRNAs in GFP-positive neurons (B)

(A[1]) Distribution of GFP-positive neurons in the spinal cord. Low magnification of the L4 segment of the spinal cord shows the highest density of GFP-positive somata and fibers in the SDH.

(A[2]–[4]) Identification of GFP-expressing neurons as GABAergic by double-staining IHC analysis with anti-GFP (A[2], green) and anti-GABA (A[3], red) in the SDH of adult GAD67-GFP knock-in mice. The GFP-positive neurons are mostly overlapped by the GABA-positive population (A[4], yellow in superimposed image). However, a few cells were positive for GABA but not for GFP (arrow). Scale bar = 50 µm in (1), 10 µm in (2)–(4).

(B) Results of RT-PCR for three GFP-positive neurons (#1–#3) sampled from the SDH are shown. NSE was used as a positive control. Lane M indicates a 100-bp DNA ladder. Values in parentheses indicate the expected length of the PCR products for NSE, GAD65, and GAD67.
Figure 2.

2-Me-5-HT-induced inward current (A) and expression of 5-HT₃ receptor mRNA (B) in a GFP-positive neuron

(A) Local application of 100-µM 2-Me-5-HT for 30 s (indicated by a horizontal bar) induced an inward current that decayed rapidly. The recording was made at a holding potential of −70 mV.

(B) After whole-cell recording, the neuron was subjected to RT-PCR detection of GAD67 and 5HT3R mRNAs. NSE was used as a positive control. Lane M indicates a 100-bp DNA ladder. Values in parentheses indicate the expected length of the PCR products for NSE, GAD67, and 5HT3R.

Figure 3.

Effects of 2-Me-5-HT on the amplitude of GABAergic IPSCs evoked by electrical stimulation

Recordings were made in the absence (A) and presence (B) of 5-HT₃ receptor-selective antagonist ONDAN at a concentration of 10 nM.

2-Me-5-HT (100 µM) was applied for 60 s, as indicated by the horizontal bar. Data points on the graph indicate the mean amplitude ± SEM derived from 12 (A) and 11 (B) neurons in the SDH. GABAergic IPSCs were evoked at 0.1 Hz. The mean control amplitude was 139.9 ± 5.0 pA in the presence of ONDAN, and not significantly different from that in the absence of the antagonist (139.1 ± 3.6 pA). Tracings in the inset are sample recordings of evoked IPSCs before (1) and during (2) the application of 2-Me-5-HT.
Figure 4.

Effects of 2-Me-5-HT on spontaneously occurring GABAergic mIPSCs

(A and B) Representative recordings showing the effects of 2-Me-5-HT on the mIPSCs recorded from a GFP-negative SDH neuron. 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 SEM of the mIPSC amplitude during a 10-s period. The tracings in the inset are sample recording spanning 20-s periods indicated by brackets before (1) and during (2) application of 2-Me-5-HT.

(C and D) Cumulative histograms of the interevent interval (C) and amplitude (D) of mIPSCs recorded from the neuron depicted in (A) and (B) before and during 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.

(E and F) Summary of the effects of 2-Me-5-HT on the frequency and amplitude of GABAergic mIPSCs. GABAergic mIPSCs were recorded from 7 neurons. The mIPSCs were collected during 90-s periods immediately before and after the start of 2-Me-5-HT application. The bars and vertical lines indicate the mean ± SEM. 2-Me-5-HT increased the frequency of the mIPSCs (E) without affecting their amplitude (F). *P < 0.05, significant; NS, not significant.
Figure 5.

Effects of 2-Me-5-HT on spontaneously occurring GABAergic mIPSCs in the presence of ONDAN

(A and B) Representative recordings showing antagonism by ONDAN of the effects of 2-Me-5-HT on mIPSCs recorded from a GFP-negative SDH neuron. The frequency (A) or amplitude (B) of the mIPSCs was not affected by 2-Me-5-HT in the presence of ONDAN. 2-Me-5-HT (100 µM) was applied for 100 s, as indicated by the horizontal bar; ONDAN (10 nM) was applied throughout the recordings, as shown by the horizontal hatched bar. The dots on the graph (A) indicate the frequency of the mIPSCs measured every 10 s. The dots and vertical bars on the graph (B) indicate the mean and SEM of the mIPSC amplitude during a 10-s period. The tracings in the inset are sample recording spanning 20-s periods indicated by brackets before (1) and during (2) application of 2-Me-5-HT.

(C and D) Cumulative histograms of interevent interval (C) and amplitude (D) of mIPSCs recorded from the neuron depicted in (A) and (B) before and during application of 2-Me-5-HT in the presence of ONDAN. The histograms labeled “control” were constructed from 69 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 60 events collected during a 90-s period immediately after the start of 2-Me-5-HT application.

(E and F) Summary of antagonism by ONDAN of the effects of 2-Me-5-HT on mIPSCs. GABAergic mIPSCs were recorded from 6 neurons. The mIPSCs were collected during 90-s periods immediately before and after the start of 2-Me-5-HT application. The bars and vertical lines indicate the mean ± SEM. 2-Me-5-HT did not affect the frequency (E) or amplitude (F) of the mIPSCs in the presence of ONDAN. NS, not significant.
Figure 6.

Effects of mCPBG on spontaneously occurring GABAergic mIPSCs in the presence or absence of TDMB

(A) Representative recordings showing the effects of mCPBG on GABAergic mIPSCs in a GFP-negative SDH neuron in the absence of TDMB. The tracings are sample records spanning 20-s periods. The tracing labeled “control” was obtained before mCPBG application and that labeled “mCPBG” was obtained during application of mCPBG (30 µM).

(B and C) Summary of the effects of mCPBG on the frequency and amplitude of GABAergic mIPSCs. The bars and vertical lines indicate the mean ± SEM (7 neurons); mCPBG increased the frequency of the mIPSCs (B) without affecting their amplitude (C). *P < 0.05, significant; NS, not significant.

(D) Representative recordings showing antagonism by TDMB of the effects of mCPBG on mIPSCs recorded from a GFP-negative SDH neuron. TDMB (10 µM) was applied throughout the recordings. The tracings are sample records spanning 20-s periods. The tracing labeled “control” was obtained in the presence of TDMB and that labeled “mCPBG+TDMB” was obtained after application of mCPBG (30 µM) in the presence of TDMB.

(E and F) Summary of antagonism by TDMB of the effects of mCPBG on GABAergic mIPSCs. The bars and vertical lines indicate the mean ± SEM (7 neurons); mCPBG did not affect the frequency (E) or amplitude (F) of the mIPSCs in the presence of TDMB. NS, not significant.
Figure 7.

Schematic showing the hypothesized mechanisms for pronociceptive and antinociceptive actions of 5-HT₃ receptors in the spinal dorsal horn.

The central neuron represents a GABAergic interneuron expressing 5-HT₃ receptors in both somadendritic regions and presynaptic terminals, as observed in our experiments.

The neuron to the left represents an enkephalinergic interneuron expressing 5-HT₃ receptors (Tsuchiya et al. 1999). Many SDH neurons reportedly coexpress GABA and enkephalin (Jo et al. 1998; Todd et al. 1992; Huang et al. 2008).

GABAergic and/or enkephalinergic neurons expressing 5-HT₃ receptors might exert postsynaptic inhibition on nociceptive projection neurons, as illustrated, or presynaptic inhibition of nociceptive transmission.

The neuron to the right represents an excitatory interneuron expressing 5-HT₃ receptors. A considerable number of the GFP-negative neurons expressing 5-HT₃ receptors, as observed in our experiments, are speculated to be excitatory in nature (Conte et al. 2005; Maxwell et al. 2003). These interneurons might be involved in the pronociceptive actions of 5-HT.
Figure 1

A

(1)

B

<table>
<thead>
<tr>
<th></th>
<th>Cell #1</th>
<th>Cell #2</th>
<th>Cell #3</th>
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<tbody>
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<td>NSE</td>
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<td></td>
<td></td>
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<tr>
<td>GAD67</td>
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<td></td>
<td></td>
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<tr>
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<td>GAD65</td>
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</tr>
<tr>
<td>M</td>
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(GAD67 231bp)
(NSE 200bp)
(GAD65 181bp)
Figure 2

A

2-Me-5-HT

B

M  NSE  5HT3R  GAD67

(GAD67 231bp)
(5HT3R 223bp)
(NSE 200bp)
Figure 3

A

B

Amplitude of GABAergic IPSC (pA) vs. Time (s)

(1) 2-Me-5-HT

(2) Ondan 2-Me-5-HT

100 ms

200 pA
Figure 4

A

B

C

D

E

F

(1)

(2)
Figure 5

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

D

![Graph D](image)

E

![Graph E](image)

F

![Graph F](image)
Enkephalinergic neuron

GABAergic neuron

Excitatory neuron

Primary Afferent

Projection neuron

5-HT
Table 1.
Primers used for single-cell reverse transcription polymerase chain reaction (RT-PCR)

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<th>Gene</th>
<th>Name</th>
<th>Primer sequence</th>
<th>Sequence start</th>
<th>Product length (bp)</th>
<th>GenBank accession number</th>
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<td>NSE-F</td>
<td>5’-ATAGTGGGGCGATGACCTGAC-3’</td>
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<td>GAD65</td>
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<td>5HT3R</td>
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<td>1390</td>
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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\textsubscript{3} receptor subunits have been cloned, namely subunit A (5-HT\textsubscript{3A}) and subunit B (5-HT\textsubscript{3B}). Although expression of 5-HT\textsubscript{3A} subunits alone yields functional 5-HT\textsubscript{3} receptors, it has been suggested that 5-HT\textsubscript{3B} subunits modify the physiological and pharmacological properties of 5-HT\textsubscript{3} receptors (Davies et al. 1999; Hapfelmeier et al. 2003). In this study, we attempted to detect the 5-HT\textsubscript{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 the primers was 20 nM each in the first PCR and 200 nM in the second PCR.
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>
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<th></th>
<th>GFP-positive neuron</th>
<th>GFP-negative neuron</th>
<th>Significance</th>
</tr>
</thead>
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<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 ± 152 (18)</td>
<td>466 ± 80 (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>
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<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>
</tbody>
</table>

Single-cell RT-PCR analysis for 5HT3R and GAD67 in GFP-positive and negative neurons that exhibited inward currents in response to 2-Me-5-HT is also shown.

* Considered to be significant in this study. Number of observations is indicated in parentheses.